ON CHEMICAL AND NUTRITIONAL DIFFERENCES BETWEEN CASEIN FROM HUMAN AND FROM COW'S MILK

By

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From the Institute of Medical Chemistry and the Pediatric Clinic,
University of Uppsala.

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Preface.

The chemical part of the present investigation has been carried out in the Institute of Medical Chemistry at Upsala. I wish to express my warmest thanks to the Head of the Institute, Prof. Gunnar Blix, M. D., who has been my teacher in medical chemistry since 1932, and who has always shown great interest in and given valuable advice concerning this work. The clinical part of the investigation has been carried out at the Pediatric Clinic of Akademiska Sjukhuset, Upsala. To the Head of this clinic, my present Chief, Prof. Curt Gyllenswärd, M. D., who has followed my work with great interest and in many ways facilitated it, I should like to offer my heartiest thanks. My sincere thanks are also due to the former Head of the clinic, Prof. Ivar Thorling, M. D., for the kind interest with which he followed the initial stages of the investigation.

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Upsala, April, 1947.

Olof Mellander.

Introduction

It is an established fact that human milk is of greater nutritional value for infants than cow's milk. This fact is well demonstrated by the lower mortality and morbidity rates for breast fed infants (cf. *e. g.* FINKELSTEIN, 1924, WOODBURY, 1926, ORKNEY, 1946). To explain this difference in nutritional value, nearly all chemical differences between the two types of milk have been taken into account. The most characteristic component of milk is casein. It is difficult, however, to attribute any part of the nutritional differences to chemical differences hitherto found between the two caseins. Exept as regards phosphorus the elementary compositions are rather similar and also the latest figures for the amino acid content (WILLIAMSON, 1944) have shown that caseins from human and cow's milk have very similar composition. It must be borne in mind, however, that proteins may be very alike as regards elementary composition and amino-acid composition, and in spite of this exhibit very different biological properties.

Such a biological difference between casein from human and cow's milk was described by WRÓBLEWSKI (1894), who found that during peptic digestion of human casein no »pseudonuclein» was formed, which is always formed when cow's casein is digested.[1] From his experiments WRÓBLEWSKI assumed that there is an essential difference in the structure of the two caseins. Since the time (1894) WRÓBLEWSKI's work was carried out, the proteolytic digestion of cow's casein has been studied by numerous investigators, while no corresponding studies on human casein have been made. For example, it has not previously been known whether ferment-resistant peptides can be isolated from proteolytic digests of human casein. Such peptides have been isolated from digests of casein from cow's milk (for references see below), and, as will be discussed later, owing to their properties they might be expected to be of special nutritional importance for the suckling.

The aim of the present investigation has been:

1) To prepare and determine the elementary composition of human casein. The analytical data in the literature on human casein are few, incomplete and, with one exception, rather old and the figures for phosphorus and sulphur given by different authors differ considerably.

2) To study the electrophoretic behaviour of casein from human and from cow's milk and to isolate electrophoretically the component with the highest phosphorus content, *viz.* the α-fraction. The author has previously shown (1939, 1945) that both caseins can be separated by means of electrophoresis into at least two different components, which have been called α- and β-casein respectively.

[1] This difference between the two caseins was also observed by v. SZCNTAGH (1893).

3) To study the breakdown of human and cow's casein when acted upon by proteolytic enzymes present in the human digestive tract, with special regard to enzyme-resistant peptides formed during hydrolysis. In the course of the work it was soon found that there are important differences in this respect in the behaviour of the two caseins.

4) To prepare enzyme-resistant peptides, *i. e.* phosphopeptones, from both caseins, in order to carry out a comparative study of some of their properties. Methods have also been worked out for the preparation of enzyme-resistant phosphopeptones from cow's casein in fairly large quantities and in a form suitable for resorption experiments on animals and infants.

Chapter I

Preparation and elementary composition of casein

Preparation of casein

For the numerous methods used for the preparation of *cow's casein*, the reader must be referred to standard texts, *e. g.* Sutermeister and Browne (1939). The cow's milk casein used in the present investigation has been prepared using a slight modification of the method of Linderström-Lang (1929). The later preparations have been carried out mainly according to Sandelin's (1943) procedure. These authors have worked out their methods very carefully and a practically ash-free casein with reproducible properties is obtained. A description of a typical preparation according to the Linderström-Lang method follows:

4 litres of unpasteurized fresh milk was centrifuged (3 000 r. p. m.) and the fat carefully removed. About 3 litres of 0.05 N hydrochloric acid was added slowly from a dropping funnel at a rate of about 1 litre per hour. The mixture was kept at room temperature and vigorous mechanical stirring applied. The hydrogen ion concentration was followed with a glass electrode and when a pH of 4.6 was reached no more acid was added. The precipitated casein was collected and washed in the centrifuge five times with distilled water, about 6 litres of water being used each time. The casein was then suspended in 2—3 litres of 60 % ethanol and the suspension stirred for half an hour. This treatment was repeated with 96 % ethanol (3 hours) and the casein was then suspended in 2 litres of 99 % ethanol and left overnight in the refrigerator. Next day, after centrifuging, the casein was washed three times with ether (2 litres each time). The casein was spread out in a thin layer on a filter paper and left in the air at room temperature for 2 hours. It was finally dried *in vacuo* for a week at a temperature of 40° C. Yield of dry casein, 115 to 145 g.

A preparation according to Sandelin is described below:

To 5 litres of fat-free fresh milk were added 274 ml of 10 % potassium oxalate solution. The milk was left in the cold (+ 2°) overnight and next morning the precipitated calcium oxalate was removed in the centrifuge. The milk was diluted with distilled water to 10 litres and 0.05 N hydrochloric acid added in the same way as

in the preparation by LINDERSTRÖM-LANG's method, described above. After the pH of the mixture had been adjusted to 4.6 the casein precipitate was collected by centrifuging, and washed with water 10 times (about 2.5 litres each time). After washing, the finely-divided casein was left standing with water overnight. The water used for washing was saturated with chloroform. The later treatment with ethanol, ether *etc.* was identical with that described above for the procedure of LINDERSTRÖM-LANG. The yield was about the same in both methods.

In order to avoid any fractionation of the casein no reprecipitation has been carried out (cf. p. 117).

The preparation of *human casein* is not as easy as that of cow's casein. The casein is in general not precipitated by the simple expedient of acidifying the milk, as is the case with cow's casein.[1] If, however, the acidified milk is heated to about 40° C and diluted several times with water, a precipitation takes place. The precipitation may also be attained by dialyzing. Following one or another of these methods several authors have prepared human casein, *e. g.* PFEIFFER (1882), DOGIEL (1885), KOBRAK (1900), ENGEL (1908), PLIMMER and LOWNDES (1937) and WILLIAMSON (1944). Another method was used by TOLMATSCHEFF (1867), MAKRIS (1876) and HOPPE-SEYLER (1883), who precipitated the casein from human milk with magnesium sulphate. WRÓBLEWSKI (1894) used ammonium sulphate for the precipitation and separated the casein from other proteins precipitated at the same time by redissolving in water and reprecipitating with acetic acid. He also dialyzed the casein for 8—14 days. LEHMAN (1894) separated the casein by filtering the milk through porous tile. The older literature concerning preparation of human casein is more fully dealt with by MAKRIS (1876) and WRÓBLEWSKI (1894).

In about half of the preparations of human casein carried out by the present author, a modification of ENGEL's procedure was used. In this method, which will be described below, the milk is diluted and, after acidifying, heated to 40° C. This seems to be a relatively gentle treatment, and the whole procedure can be performed in about 36—48 hours. In the later preparations a method was used similar to that of SANDELIN for cow's casein. In this case the milk is treated with potassium oxalate before being acidified. There are no difficulties in obtaining a precipitation of the casein when the hydrogen ion concentration is brought to the isoelectric region.

A typical preparation using the modified method of ENGEL is described below:

Fresh human milk[2] was centrifuged until free from fat. Samples collected from day to day were kept in the frozen state until a sufficient quantity of milk had been

[1] This was pointed out already by BERGIUS (1772) and since than by nearly all who have prepared human casein. Only in two cases, LANGSTEIN and EDELSTEIN (1910) and BOSWORTH and GIBLIN (1918), human casein seems to have been prepared only by acidifying fat-free human milk.

[2] All human milk used in this investigation was mixed milk from several women.

collected. To 4 litres of milk were added about 40 litres of distilled water; 4 litres of ca. 0.1 N acetic acid were then added slowly, stirring continually, the temperature being kept at about 20° C; ca. 0.1 N sodium acetate was then added until a pH of 4.6 was reached (3.2 litres).[1] The hydrogen ion concentration was measured with a glass electrode. The mixture was heated to 35—40° C for one hour and then placed overnight in the refrigerator at a temperature of + 2°. During the night the fine precipitate of casein formed sediments at the bottom of the vessel. The following morning the supernatant, practically clear, liquid was siphoned off and the precipitate collected and washed four times with 4 litres of acetate buffer of the same composition as used for the precipitation. The precipitate was then washed twice with distilled water (2 litres), 96 % ethanol (twice, 2 litres each time), absolute ethanol (0.5 litre) and finally with ether (one litre). The casein was dried in the same way as described for cow's casein. The usual yield of casein with this method was about 15 g from 4 litres of milk.

In the later preparations the following method was used:

To 5 litres of fat-free human milk were added 135 ml of 10 % potassium oxalate. After standing overnight in the cold (+ 2°) the calcium oxalate formed was removed by centrifuging. 5 litres of water was added and 0.05 N hydrochloric acid was then slowly stirred in until the pH of the solution was 4.6. The solution was heated to 40° C for one hour and left overnight in the refrigerator at + 2°. Next morning the supernatant liquid was siphoned off, the precipitate isolated in the centrifuge and washed with water until free from chlorine (usually 3—5 washings with one litre each time were sufficient). The washings with ethanol and ether were carried out as in the preparation described above. The yield of casein with this method was 20—25 g from 4 litres of human milk.

The yield of human casein is thus appreciably higher when the second method is used.

Elementary composition of casein

Casein from cow's milk has been analyzed by several authors. One of the earliest and most quoted of them is HAMMARSTEN (1883). In Table 1 some of the analytical figures obtained by different authors have been collected. Human milk casein has not been so extensively investigated in this respect. In Table 2 the results hitherto reported (as far as the author knows) are collected.

The most extensive of the earlier studies is that of WRÓBLEWSKI (1894), whose figures are the mean value of 5 different preparations. The figures of the other authors are generally based on the analysis of a single preparation. It may be seen from the table that the greatest variations between the preparations of the different authors are found for phos-

[1] The optimal hydrogen ion concentration for the precipitation of casein from human milk has been studied by several authors with very different results. YLPPÖ (1913) found an optimal precipitation at a pH of 4.16, DEMUTH (1924) found with acetic acid-sodium acetate an optimum at pH 4.68, SCHEMANN (1928) obtained the most complete precipitation at pH 4.6 and WANG and WOOD (1930) at pH 4.97. MEYER (1925, 1926) and TRENDTEL (1927) found that the precipitation optimum was not the same in milk from different women.

Table 1. *Elementary analyses of casein from cow's milk according to different authors.*

Author	Year	C %	H %	N %	P %	S %	O % (by difference)
Hammarsten	1883	52.96	7.05	15.65	0.85	0.72	(22.78)
Chittenden, Painter	1885—1888	53.30	7.07	15.91	0.87	0.82	(22.03)
Lehman, Hempel	1894	54	7.04	15.6	0.85	0.77	(21.74)
Burow	1905	52.83	7.10	15.64	0.81	0.73	(22.91)
Tangl	1908	52.69	6.76	15.65	0.88	0.83	(23.14)
Van Slyke, Bosworth	1913	53.50	7.13	15.80	0.71	0.72	(22.08)
Bleyer, Seidl	1922			15.52	0.83	0.75	
Sandelin	1943			15.54	0.84		
Ramsdell, Whittier[1]	1944	52.88	7.00	15.51	0.79	0.77	(23.05)

[1] Casein separated from milk by means of the Sharples super-centrifuge.

Table 2. *Elementary analyses of casein from human milk according to different authors.*

Author	Year		C %	H %	N %	P %	S %	O % (by difference)
Makris[1]	1876		52.35	7.27	14.65	—	25.73 (S+O)	
Wróblewski	1894		52.24	7.32	14.97	0.68	1.12	(23.66)
Lehman, Hempel[1]	1894		—	—	—	—	1.09	—
Langstein, Bergell[1]	1908	I	53.01	7.14	14.60	0.25	0.71	—
		II	52.63	6.94	14.34	0.27	0.85	—
Langstein, Edelstein	1910		52.7	7.10	14.04	0.24	0.33	—
» [2]	»		53.6	7.19	14.5	0.22	0.67	—
» [3]	»		51.56	7.22	15.0	0.22	0.65	—
» [4]	»		52.15	6.77	14.8	0.29	0.76	—
			52.52	7.08	14.7	0.24	0.84	—
Bosworth, Giblin[1]	1918		—	—	15.75	0.70	0.70	—
Plimmer, Lowndes[1, 5]	1937		—	—	14.70	0.45	0.79	—
			—	—	14.37	0.45	0.78	—
Williamson	1944		—	—	14.90	—	—	—

[1] Values most probably not calculated on an ash-free basis.
[2] Casein prepared according to Kobrak (1900).
[3] » » » » » » , after 5 reprecipitations.
[4] » » » » Engel (1908).
[5] The values given were obtained on a mixture of many different preparations. In the separate preparations the N values varied between 13.96 and 14.98, the P values between 0.41 and 0.73 and the S values between 0.42 and 1.02.

Table 3. *Elementary analyses of cow's casein (author's preparations).*

(LL = preparation according to Linderström-Lang,
S = preparation according to Sandelin.)

Preparation No.	Method of preparation	C %	H %	N %	P %	S %	O %	Per cent analyzed
V	LL	53.57	7.13	15.66	0.81	0.64	23.24	101.05
VI	LL	53.08	6.95	15.65	0.85	0.81	22.59	99.93
X	S	53.05	7.05	16.08	0.76	0.88	22.16	99.98
XVI	S	52.90	6.91	15.25	0.76	0.73	22.87	99.42
XVIII	S	—	—	15.05	0.81	—	—	—

Table 4. *Elementary analyses of human casein (author's preparations).*

(E = preparation according to Engel,
S = preparation according to Sandelin.)

Preparation No.	Method of preparation	C %	H %	N %	P %	S %	O %	Per cent analyzed
13	E	53.78	7.12	15.14	0.47	0.65	22.63	99.79
15	E	53.73	7.20	15.11	0.47	0.65	22.31	99.47
19	E	53.73	7.13	14.83	0.47	0.67	22.94	99.77
20	E	—	—	15.16	0.51	—	—	—
36	E	53.50	7.14	15.18	0.46	—	—	—
38	S	53.06	6.97	15.16	0.47	1.13	22.01	98.80
39	S	53.55	7.04	14.89	0.35	1.24	22.53	99.60
40	S	53.17	7.00	14.93	0.32	1.25	22.49	99.16
49[1]	S	52.47	6.83	14.52	0.42	—	22.98	—
57	S	—	—	—	0.45	—	—	—

[1] Corrected for an ash content of 1.4 %.

phorus and sulphur. In WRÓBLEWSKI's 5 preparations the lowest phosphorus value is 0.63 and the highest 0.80 %, for sulphur the corresponding figures are 0.86 and 1.53 %. WRÓBLEWSKI compares his figures for human casein with the corresponding values for cow's casein according to HAMMARSTEN, and states that the carbon, nitrogen and phosphorus content is lower and the hydrogen and sulphur content higher in human than in cow's casein. This is a proof, he says, that the two caseins are different substances. Remarkably low phosphorus values were obtained by LANGSTEIN and BERGELL (1908). The casein was, however, probably partly decomposed, as their preparation I was obtained by "Selbstsäuerung" of human milk, and preparation II was precipitated by adding acetic acid and sodium chloride after removing the first precipitate

Table 5. *Sulphur content of different preparations of human casein.*

Preparation No.	Method of preparation	S %
5	E	0.47
13	E	0.65
15	E	0.65
19	E	0.67
28	E	0.61
38	S	1.13
39	S	1.24
40	S	1.25
41	S	1.08
42	S	1.22
53	S	1.26

(preparation I). In a later work LANGSTEIN and EDELSTEIN (1910) prepared human casein by precipitating human milk freed from fat in the centrifuge with acetic acid, without previously diluting the milk. It was, however, not always possible to get a precipitation in this way. After washing with water, ethanol, and ether, the casein was »im Soxhlet Apparat völlig vom Fett befreit». The authors also prepared casein according to the methods of ENGEL and of KOBRAK, but in both cases a Soxhlet extraction with ether, as in the original procedure of KOBRAK, was performed. A preparation according to KOBRAK was also reprecipitated 5 times with acetic acid and sodium chloride from solution in aqueous ammonia. As is seen from Table 2 all preparations obtained by LANGSTEIN and EDELSTEIN show phosphorus values below 0.30 %. PLIMMER and LOWNDES (1937) found that different preparations of human casein had a very different elementary composition. The values for nitrogen varied between 13.96 and 14.98 %, for phosphorus between 0.41 and 0.73 % and for sulphur between 0.64 and 1.02 %. The values for phosphorus obtained when several preparations were mixed (see Table 2) were 0.45 % which is in good agreement with the author's values.

Part of the casein preparations used in the present investigation have been analyzed for the elements shown in Tables 3 and 4. Standard micro-analytical methods were employed in each case. Carbon and hydrogen were determined by a modification of FRIEDRICHS' (1932) method worked out by W. Kirsten in this laboratory. For nitrogen the common micro-modification of KJELDAHL's method (cf. PREGL-ROTH 1935) was used. Phosphorus was determined according to TEORELL (1931). Sulphur has been determined by the method of GROTE and KREKELER (1933) as modified by SCHÖBERL (1937). Oxygen, finally, was determined by

Unterzaucher's (1940) micro-modification of Schütze's method (1939).[1]

For analysis, the casein preparations were dried to constant weight *in vacuo* over phosphorus pentoxide at 100° C. Most of the preparations have been analyzed for calcium by the method of Widmark and Vahlquist (1930) and only in one case (preparation No. 49 of human casein) was calcium found to be present in an amount exceeding 0.1 %. Except for preparation No. 49 therefore, no correction for ash content has been considered necessary. As mentioned above both Linderström-Lang and Sandelin report that cow's casein prepared by their methods is practically free from ash.

Discussion and Summary of Chapter I

Human casein has been prepared by two different methods. The main differences between the methods of preparation are that one procedure involves a greater dilution of the milk, that acetic acid is used for the precipitation instead of hydrochloric acid, and that the inorganic calcium salts of the milk are not removed by potassium oxalate. The preparations according to Engel have a very constant elementary composition, while those according to Sandelin show minor differences between the analytical figures. Both methods give products which do not differ very much from each other, except as regards the sulphur content. The phosphorus values now obtained are in good agreement with the values given by Plimmer and Lowndes (1937) for their mixed preparations but are considerably lower than those given by Wróblewski (1894) and Bosworth and Giblin (1918) and higher than those reported by Langstein and Edelstein (1910). Most of the preparations of human casein now analyzed show a phosphorus content of around 0.47 %. Two samples (both prepared according to the modified Sandelin procedure) have given lower values. The hydrogen and nitrogen figures obtained agree fairly well with values previously published by other authors, but the carbon values are slightly higher.

As regards sulphur (cf. Table 5), it is remarkable that part of the preparations of human casein contain around 1.2 % and the other part only half as much, *i. e.* 0.6 %. The higher figures have always been obtained for preparations made with Sandelin's method, and the lower values have, on the other hand, been obtained when the preparations were

[1] All carbon, hydrogen, sulphur and oxygen analyses have been performed in the micro-analytical laboratory at the Institute of Medical Chemistry by Mr W. Kirsten. Part of the nitrogen and phosphorus analyses have been carried out by Mr E. Lindberg and by the author. The analytical errors involved are less than one per cent in all methods with the exception of the phosphorus determination, where the error is within 2 % (cf. Teorell 1931).

carried out according to ENGEL's method. The difference in sulphur content may be due to loss of methionine or cystine from the molecule. It may be mentioned that SULLIVAN (1929) has found that cow's casein from different sources can have very different cystine content (0.15—0.50 %), and that JONES and GERSDORFF (1934) have shown that reprecipitation of casein successively lowers the cystine content. For example one preparation initially containing 0.336 % of cystine, contained after five reprecipitations only one tenth of this value. If a splitting-off of amino-acid sulphur was behind the lower sulphur content of the preparations according to ENGEL, it is not easy to understand why such a splitting-off should occur more easily with this method than when the other method is used, especially as the former gives products whose elementary composition is so constant.

Another possible reason for the difference in sulphur content might be that some compound containing a high percentage of sulphur is precipitated out together with the casein. In this connection it must be recalled that WRÓBLEWSKI reported the presence of a protein, "opalisin", in human milk. According to WRÓBLEWSKI this substance has the following elementary composition: C 45.01, H 7.31, N 15.07, P 0.80, S 4.70, (O 27.11) %. The high content of sulphur must be noted. However, after WRÓBLEWSKI, nobody appears to have isolated this protein. In order to double the sulphur content of 0.6 % found in the preparations according to ENGEL, an amount of "opalisin" of about 13 g per 100 g of casein has to be present in the preparations according to SANDELIN. This would give an extra protein component in the electrophoretic diagrams, provided of course that its mobility is not the same as that of one of the casein components. The casein preparations with higher sulphur content have in no case given an extra peak in the electrophoretic diagrams. The author considers, therefore, that the higher sulphur values are not likely to depend on the presence of "opalisin" or some other protein with a high sulphur content.

Owing to lack of suitable methods for the determination of oxygen, no oxygen analyses on casein have hitherto been made. The recent development by UNTERZAUCHER (1940) of a micromethod for the determination of oxygen has now for the first time permitted a complete analysis of the two types of casein.

Tables 3 and 4 show that all preparations of human casein have a phosphorus content only about half of that found in cow's casein. With regard to the content of hydrogen and oxygen there are only minor differences. The figures for carbon are somewhat higher and those for nitrogen somewhat lower for human casein than for cow's casein.

Chapter II

The electrophoretic inhomogeneity of casein

Review of literature: The question of the inhomogeneity of cow's casein was first raised by the work of Danilewski and Radenhausen (1880), who considered cow's casein to be a mixture of two different proteins. Their method of separation involved treatment with hot ethanol. The components isolated differed *inter alia* with regard to sulphur content. Hammarsten (1883, 1885) reinvestigated the matter very carefully and criticized their view. Osborne and Wakeman (1918, a, b) obtained from the alcoholic washings from large quantities of casein a substance with the following elementary composition: C 54.91, H 7.17, N 15.71, S 0.95, P 0.08 %. They were of the opinion that their substance was quite different from casein.

Kondo (1925) and Linderström-Lang and Kodama (1925) showed that when casein was dissolved in hydrochloric acid the amount dissolved was not independent of the amount undissolved. This result indicated that casein from cow's milk is not a homogeneous protein. Linderström-Lang (1929) fractionated cow's casein by treatment with very dilute alcoholic hydrochloric acid. He obtained no less than seven different fractions with different elementary composition as regards phosphorus. Linderström-Lang is of the opinion, however, that these seven fractions are not all present as components of native casein. Demanez (1933) has investigated the serological properties of casein fractions prepared according to Linderström-Lang, but was unable to find any serological difference. Since the publication of Linderström-Lang's work a large number of methods for the fractionation of casein have been described in the literature (cf. Sutermeister and Browne, 1939). The fractions obtained by most of these methods have probably not been homogeneous, however.

Svedberg, Carpenter and Carpenter (1930) found that cow's casein prepared according to Hammarsten (1910) or according to Van Slyke and Baker (1918) is heterogeneous in the ultracentrifuge, and that different preparations obtained with the method of Van Slyke and Baker were not identical. A fraction of the casein soluble in slightly acid 70 % ethanol behaved as a homogeneous substance, however, having a molecular weight of 375 000. Carpenter and Hucker (1930) do not consider this fraction identical with that described by Osborne and Wakeman. They fractionated casein by means of 70 % ethanol and potassium oxalate into three different fractions with molecular weights of 98 000, 188 000 and 375 000 respectively. These three fractions showed distinct serological differences. The fraction soluble in 70 % ethanol (mol. weight 375 000) was also serologically different from the fraction

obtained with Osborne and Wakeman's method. PHILPOT and PHILPOT (1939) found that the sedimentation constant of casein depended on the calcium content of the casein solution.

A more definite picture of the inhomogeneity of casein was first obtained by electrophoresis in the TISELIUS (1937) apparatus. The present author showed some years ago (1939) that cow's casein prepared according to LINDERSTRÖM-LANG's method was electrophoretically inhomogeneous. In the electrophoresis diagrams three peaks were observed, and these were assumed to correspond to three different casein fractions, which in the order of decreasing mobility were named α-, β- and γ-casein respectively. The α-fraction was isolated in the electrophoresis apparatus and was found to have a higher content of phosphorus than the original casein. The comparatively high electrophoretic mobility was attributed to a higher negative charge caused by the higher content of phosphoric acid. The β-fraction is difficult to isolate in the electrophoresis cell, and was therefore not obtained in pure form at the time. An attempt to isolate the γ-fraction was made, but the protein concentration in the solution obtained was very small. This fact, together with the low mobility, makes it possible that this peak of the electrophoretic diagram is due to boundary anomalies (the so-called δ and ε effects, cf. SVENSSON (1946)). The latter opinion is held by WARNER (1944) who has also worked out a method for separating α- and β-casein by isoelectric precipitation in dilute solutions at different pH. WARNER has also isolated the "γ-fraction" in the electrophoresis apparatus, and in agreement with the author found that it contains a small amount of protein. According to WARNER the protein concentration found only corresponds to about 4 % of the area of the γ-peak in the electrophoresis diagram. For two different preparations of α-casein WARNER found a phosphorus content of 0.99 and 1.00 % respectively, as against a phosphorus content of the total casein of 0.85 and 0.87 % respectively. These figures agree very well with those previously published by the author (1939) (0.96 % for α-casein and 0.81 % for total casein). For casein from human milk, no investigations regarding the homogeneity were published until the author's electrophoretic experiments, preliminarily reported in 1945.

AUTHOR'S EXPERIMENTS

Electrophoretic behaviour of cow's and human casein

All experiments were performed in the TISELIUS apparatus (1937) equipped with the diagonal slit optical system of SVENSSON (1946). Figures 1 and 2 show the electrophoretic diagrams for cow's and human casein respectively at different pH, using a protein concentration of about 0.5 % and buffers with an ionic strength of 0.15. On the alkaline side of the isoelectric region the electrophoresis diagrams for both types

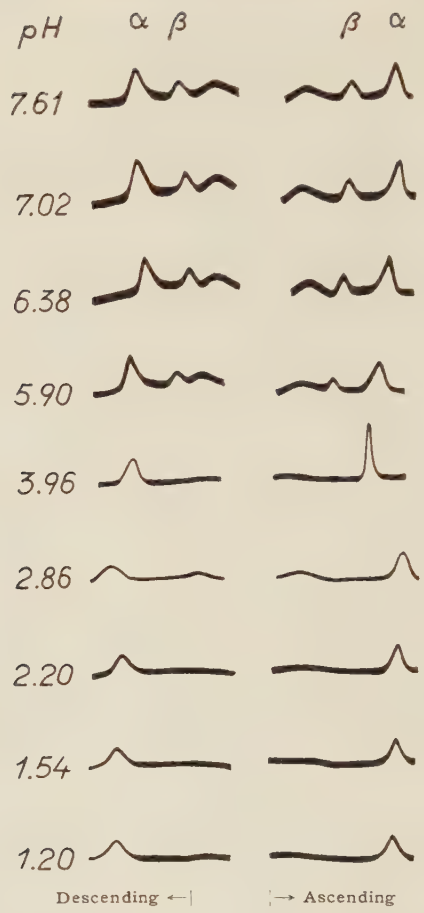

Fig. 1. Electrophoretic patterns of cow's casein at different pH. Ionic strength 0.15 (phosphate, acetate or glycine 0.10 + sodium chloride 0.05). Potential gradient about 5 volts/cm. Concentration of casein about 0.5 % (at pH 3.96 < 0.5 %, see p. 128).

of casein show distinct α- and β-peaks. On the acid side, however, the β-peak of cow's casein is diffuse and but slightly visible on the photographic plates. Figs. 3 6 show that similar electrophoretic diagrams are also obtained at higher protein concentrations and also at an ionic strength of 0.25. WARNER (1944) has under certain conditions (protein concentration between 0.6 % and 1 % and veronal buffers) observed a division of the α-peak into two peaks. No such division has been observed in the course of the present work, in which veronal buffers have

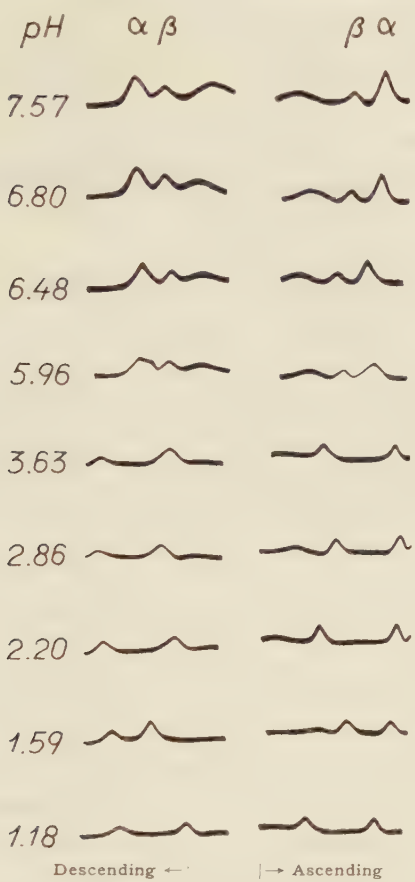

Fig. 2. Electrophoretic patterns of human casein at different pH. Ionic strength 0.15 (phosphate or glycine 0.10 + sodium chloride 0.05). Potential gradient about 5 volts/cm. Concentration of casein about 0.5 %.

not been used. The mobilities as a function of pH over the range pH 1—8 of the different fractions of cow's and human caseins have been studied. The results are shown in Figs. 7 and 8 and in Table 6. In these experiments the protein concentration has been held at 0.5 % and the ionic strength of the buffer adjusted to a value of 0.15. The mobility values of the β-peak of cow's casein in the acid pH range are rather uncertain owing to the smallness and diffuse nature of the peaks observed. On the alkaline side of the isoelectric region phosphate buffers were used. The buffers used on the acid side of the isoelectric region were sodium acetate-acetic acid and glycine-hydrochloric acid respectively.

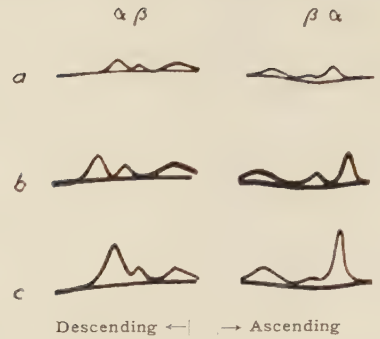

Fig. 3. Electrophoretic patterns of human casein at different concentrations (a 0.25 %, b 0.5 %, c 1.0 %). Phosphate buffer of pH 7.60 and ionic strength 0.15 (phosphate 0.10 + sodium chloride 0.05). Potential gradient about 5 volts/cm.

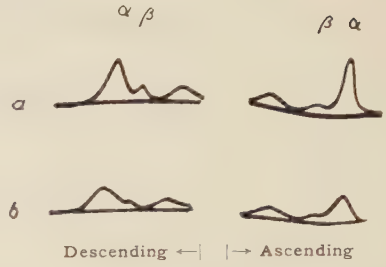

Fig. 4. Electrophoretic patterns of human casein at different ionic strength (a 0.15, b 0.25) of phosphate buffer (phosphate 0.10 + sodium chloride 0.05 and 0.15 respectively), pH 7.60. Protein concentration ca. 1 %. Potential gradient about 5 volts/cm.

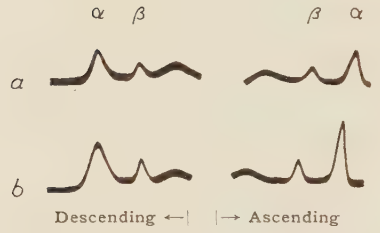

Fig. 5. Electrophoretic patterns of cow's casein at different concentrations (a 0.5 %, b 1.0 %). Phosphate buffer of pH 7.60 and ionic strength 0.15 (phosphate 0.10 + sodium chloride 0.05). Potential gradient about 5 volts/cm.

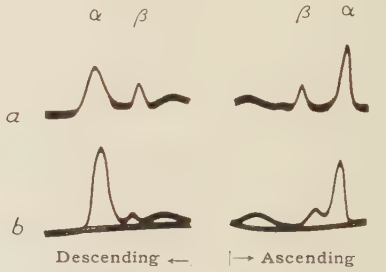

Fig. 6. Electrophoretic patterns of cow's casein at different ionic strength (a 0.15, b 0.25) of phosphate buffer (phosphate 0.10 + sodium chloride 0.05 and 0.15 respectively), pH 7.60. Protein concentration ca. 1 %. Potential gradient about 5 volts/cm.

In each case buffers of the desired pH and an ionic strength of 0.10 were prepared and sodium chloride added to bring the ionic strength to 0.15. The casein solutions were prepared as described on p. 139 and diluted to a protein concentration of 0.5 % with the buffer to be used for electrophoresis. They were dialyzed for 48 hours against the same buffer. The time of electrophoresis varied between one and three hours.

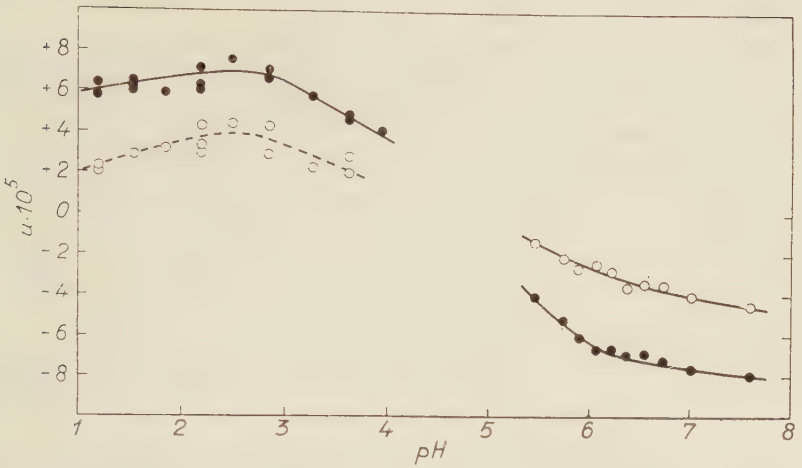

Fig. 7. Electrophoretic mobilities of α- and β-casein from cow's milk at different pH. Experimental conditions are given in Table 6. (α-casein ●, β-casein ○).

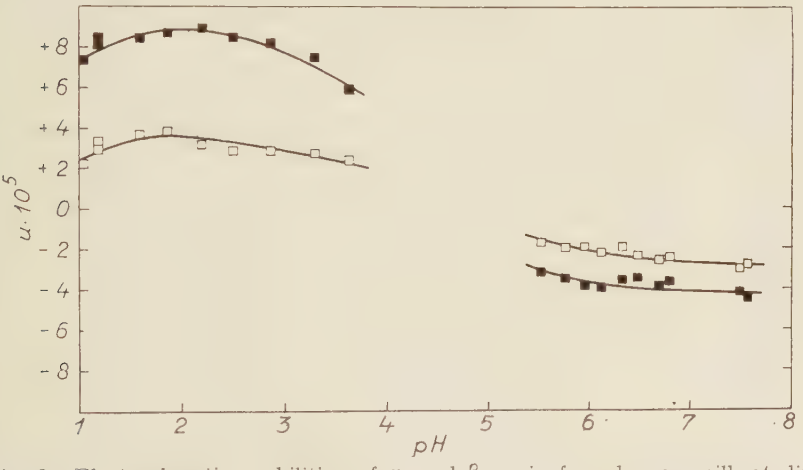

Fig. 8. Electrophoretic mobilities of α- and β-casein from human milk at different pH. Experimental conditions are given in Table 6. (α-casin ■, β-casein □).

Electrophoretic separation of α-casein

As already mentioned the author has previously shown (1939) that α-casein from cow's milk has a higher content of phosphorus than total casein. In the course of the present work a number of electrophoretic separations of α-casein both from cow's and human milk have been car-

Table 6. *Electrophoretic mobilities, in cm^2 volt ^{1} $sec.^{-1} \times 10^5$, of cow's and human casein fractions. Ionic strength 0.15 (phosphate, acetate or glycine 0.10 + sodium chloride 0.05). Potential gradient about 5 volts/cm. Concentration of casein about 0.5 % (at pH 3.96 < 0.5 % see p. 128). Temp. 0.0° C. Mobilities measured on the ascending side.*

pH	Buffer	Cow's casein			Human casein		
		α	β	γ	α	β	γ
7.61	Phosphate	−7.9	−4.5	−0.9			
7.57	»				−4.5	−2.8	−0.6
7.50	»				−4.2	−3.0	−0.5
7.02	»	−7.6	−4.1	−0.7			
6.80	»				−3.7	−2.5	−0.2
6.74	»	−7.3	−3.6	−0.6			
6.69	»				−4.1	−2.6	−0.2
6.55	»	−6.9	−3.4	−0.6			
6.48	»				−3.5	−2.4	−0.2
6.38	»	−7.0	−3.7	−0.3			
6.32	»				−3.6	−1.9	−0.1
6.13	»				−4.0	−2.2	−0.2
6.08	»	−6.7	−2.5	−0.2			
5.96	»				−3.8	−1.9	−0.3
5.90	»	−6.2	−2.8	−0.2			
5.76	»				−3.5	−2.0	−0.3
5.75	»	−5.3	−2.2	±0.0			
5.52	»				−3.2	−1.8	—
5.47	»	−4.2	−1.5	−0.2			
3.96	Acetate	+4.0	—	—			
3.64	Glycine	+4.9	(+2.8)	—			
3.64	»	+4.6	(+2.0)	—			
3.63	»				+5.9	+1.4	—
3.28	»	+5.8	(+2.2)	—	+7.5	+2.7	—
2.86	»	+6.6	(+2.9)	—	+8.2	+2.9	—
2.50	»	+7.5	(+4.4)	—	+8.4	+2.9	—
2.20	»	+7.1	(+4.3)	—			
2.20	»	+6.2	(+3.3)	—			
2.20	»	+6.3	(+3.0)	—	+8.9	+3.1	—
1.85	»	+5.9	(+3.2)	—	+8.7	+3.8	—
1.59	»				+8.4	+3.7	—
1.54	»	+6.1	(+2.9)	—			
1.54	»	+6.5	—	—			
1.54	»	+6.3	--	—			
1.20	»	+5.8	(+2.1)		+8.2	+2.9	—
1.20	»				+8.4	+3.4	—
1.04	»				+7.3	+2.3	—

Mobility values within brackets are uncertain.

Table 7. *Analytical data on casein and α-casein from cow's milk.*

Cow's casein preparation No.	Total casein			α-casein		
	% N	% P	N P (atomic ratio)	mg N/ml	mg P/ml	N/P (atomic ratio)
II[1]	15.20	0.83	40.6	0.499	0.032	34.5
III[1]	14.64	0.76	42.7	0.193	0.118	36.2
V	15.66	0.81	42.8	0.491	0.039	27.9
XVI	15.25	0.76	44.4	0.803	0.055	32.3

[1] The analytical figures for this casein preparation are taken from an earlier publication of the author (1939).

Table 8. *Analytical data of casein and α-casein from human milk.*

Human casein preparation No.	Total casein			α-casein		
	% N	% P	N/P (atomic ratio)	mg N/ml	mgP/ml	N/P (atomic ratio)
13	15.14	0.47	71.3	0.778	0.0311	55.5
				0.755	0.0305	54.8
				0.495	0.0293	37.4
				0.476	0.0380	27.7
15	15.11	0.47	71.2	0.558	0.0388	31.0
				0.381	0.0217	38.9
36	15.18	0.46	73.1	0.453	0.0437	23.0
				0.402	0.0418	21 3
				0.268	0.0137	43.3

Fig. 9. Electrophoretic pattern of α-casein from cow's casein, separated in the preparative apparatus of Svensson. Phosphate buffer of pH 7.60 and ionic strength 0.15. Protein concentration ca. 0.5 %.

ried out. The separations have been performed in the Tiselius (1937) apparatus, in which case it was necessary to collect the α-fraction from many different runs in order to get a reasonable quantity of material. The α-fraction from cow's casein was also obtained by continous electrophoresis in the large preparative electrophoresis apparatus of Svens-

SON (1946).[1] In these experiments a 3 % solution of casein in 0.1 N sodium bicarbonate[2] (pH ca. 7.5) was used, and sodium bicarbonate solution of the same concentration was used also as external buffer in the electrophoresis. The α-fractions obtained on analysis gave the results shown in Table 7.

In order to check the homogeneity of the electrophoretically isolated α-casein, the latter was subjected to a new electrophoresis experiment using phosphate buffer of pH 7.60 and ionic strength 0.15. The corresponding diagram given in Fig. 9 shows only one definite component.

α-casein from human milk has been isolated in an analogous manner using TISELIUS' (1937) apparatus. For analytical data see Table 8. The variations in the N/P ratios for human casein, obtained in different separations from the same casein preparations, are probably due to the fact that the isolation of the α-fraction in the electrophoresis apparatus is technically difficult, owing to the small difference in mobility of the α- and β-fraction of human casein.

An attempt to extend Warner's method for the preparation of α-casein from cow's casein to the preparation of human α-casein

α-casein was prepared by WARNER'S (1944) method from 150 g of cow's casein. Six precipitations were required to get an electrophoretically homogeneous substance (cf. Fig. 10). On analysis this preparation gave N, 15.73 %, and P, 0.90 %, as compared with 15.25 and 0.76 % respectively for the original casein. Another preparation was made in the same manner from cow's casein. In this case the electrophoretically homogeneous α-casein obtained gave on analysis N, 15.48 %, P, 0.88 %, compared with 15.13 and 0.79 % respectively for the original casein. The electrophoretic mobilities of this α-casein in the pH range 6—8 are given in the diagram shown in Fig. 11 and also in Table 9.

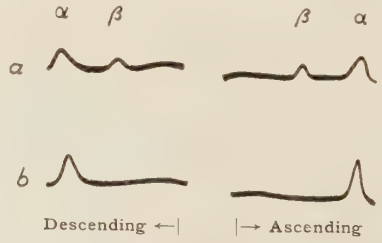

Fig. 10. Electrophoretic pattern of α-casein (b) from cow's casein (a), separated according to WARNER. Phosphate buffer of pH 7.60 and ionic strength 0.15. Protein concentration ca. 0.5 %.

[1] The author is indebted to Docent H. SVENSSON for kind help in this experiment.
[2] A buffer free from phosphorus had of course to be used in these experiments.

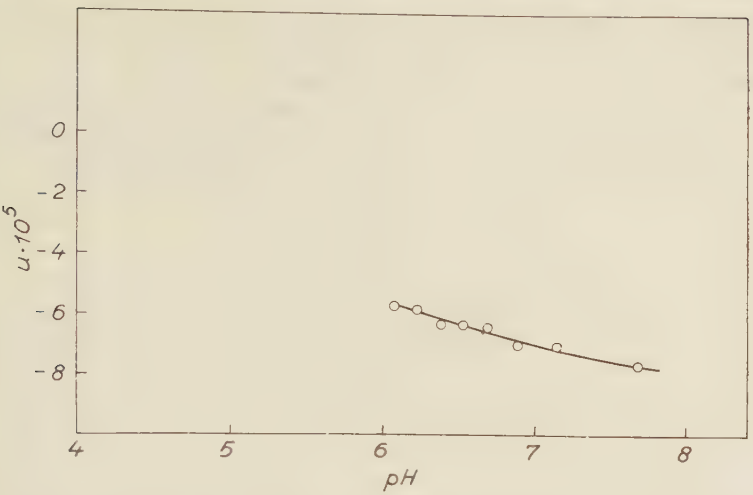

Fig. 11. Electrophoretic mobility of α-casein, prepared from cow's casein according to WARNER, at different pH. Experimental conditions are given in Table 9.

Table 9. *Electrophoretic mobilities, in* $cm^2\ volt^{-1}\ sec.^{-1} \times 10^5$, *of α-casein prepared from cow's casein. Ionic strength 0.15 (phosphate 0.10 + sodium chloride 0.05). Potential gradient about 5 volts/cm. Concentration of α-casein 0.5 %. Temp. 0.0° C.*

pH	Mobility
7.68	−7.69
7.14	−7.01
6.88	−7.01
6.69	−6.47
6.53	−6.45
6.38	−6.41
6.23	−5.96
6.07	−5.79

An attempt was then made to prepare α-casein from human casein in an analogous manner. Twenty grammes of human casein (mixture of different preparations) were dissolved in water (6 litres) with the aid of 80 ml N/10 sodium hydroxide. The pH of the resulting solution was about 6.5. The solution was added to 80 ml of 0.3 N hydrochloric acid. The pH of the resulting solution was 2.2; 0.05 N sodium hydroxide was now added until the pH was 4.2. The solution was heated to 40° C for one hour. The resulting precipitate was collected and washed twice with water, followed by ethanol and ether in the usual manner. The dry

precipitate weighed 1.4 g, and gave on analysis 0.37 % P as compared with 0.45 % for the untreated casein.

Another attempt using the same procedure gave a similar result. The electrophoretic diagram of the dissolved first precipitate gave no evidence of any fractionation. It is thus evident that WARNER'S original procedure is not suitable for preparation of α-casein from human casein.

Discussion and Summary of Chapter II

The electrophoretic behaviour of human and cow's casein has been studied. The author has previously found that the electrophoretic diagram of cow's casein shows three distinct peaks when phosphate buffers are used in electrophoresis. The casein fractions corresponding to the two fastest moving peaks were designated α- and β-casein respectively. It has not yet been settled whether the γ-peak corresponds to a real casein component. The fact that the γ-peak is not appreciably diminished with increasing ionic strength of the buffer (Figs. 4 and 6) makes it probable that it is not due to boundary anomaly effects (ϑ and ε effects) only. The mobility of the γ-peak is very low, however, and nearly independent of the hydrogen ion concentration. On the other hand, the solution in the electrophoretic tube behind the β-peak on the descending side no doubt contains a small amount of protein, as indicated by the positive sulphosalicylic acid test. The mobilities of the α- and β-components of human and cow's casein have been determined on both sides of the isoelectric region. Phosphate buffers were used in the alkaline range, and glycine and acetate buffers in the acid range. On the alkaline side the α-component of cow's casein shows somewhat higher mobility than that of human casein, but on the acid side the opposite is the case. There is no pronounced difference between the mobilities of the β-components of the two caseins. A remarkable fact is that on the acid side of the isoelectric region (acetate and glycine buffers) the β-peak of cow's casein is only very slightly developed, in contrast to the β-peak of human casein. The reason for this behaviour of cow's casein on the acid side of the isoelectric region is not clear. In view of the fact that WARNER has found a low solubility of isolated cow's β-casein in certain acid buffers (lactate of pH 3.07 and ionic strength 0.1), it should perhaps be pointed out that the casein solutions used in the author's experiments were always quite clear, except at pH 3.96 (acetate buffer). In this case a precipitate occurred which had to be removed by centrifugation before electrophoresis. The peculiar behaviour of the β-peak is probably due to the complex formation between the α- and β-components studied by KREJCI *et al.* (1941, 1942) and by WARNER. For a detailed discussion of this behaviour of cow's α- and β-casein reference must be made to WARNER (1944).

The difference in mobility between α- and β-casein on the alkaline side of the isoelectric region is considerably greater for the two components of cow's casein than for those from human casein. This makes it sometimes difficult to get a satisfactory separation of human α- and β-casein in an apparatus with ordinary cells, especially in experiments in which the ionic strength is higher than 0.15. The mobility diagrams and Table 6 also show that in the most acid region the mobilities tend to decrease. For cow's casein this decrease begins at about pH 2.2, and for human casein at about pH 1.8.

α-casein from human and cow's casein has been isolated electrophoretically and has in both cases been found to have a lower nitrogen-phosphorus ratio than the original casein. For cow's casein, this has been previously observed by the author and confirmed by WARNER. In human casein a relatively greater amount of the total phosphorus is present in the α-fraction.

α-casein from cow's casein has also been prepared by the isoelectric precipitation method of WARNER. This method was found unsuitable for human casein. Cow's α-casein prepared by WARNER's method had a nitrogen-phosphorus ratio agreeing with that for electrophoretically isolated α-casein. This α-casein was also analyzed for serine according to the method of BOYD and LOGAN (1942), and was found to have a considerably higher serine content (6.8 %) than the original casein (3.5 %).

CHAPTER III

The hydrolysis of casein by proteolytic enzymes

Review of literature. Unlike most other proteins, cow's casein cannot be completely broken down by the action of proteolytic enzymes of the digestive tract. Part of the casein molecule is stable vis-à-vis such enzymes, and it has been known for a long time that this part of the casein contains considerable quantities of phosphorus. It was suggested many years ago by SALKOWSKI (1893) that the organically bound phosphorus, formed during peptic digestion of casein was, perhaps, of special importance for the nutrition of the suckling. SALKOWSKI expresses his views as follows:

"Solange man annimmt, das der Phosphor des Caseins bei der Verdauung als Paranuclein unlöslich abgespalten wird, is nicht wohl einzusehen, zu welchem Zweck das Casein phosphorhaltig ist — es müsste denn das Nuclein im Darmkanal gespalten werden. Entstehen dagegen lösliche phosphorhaltige Verdauungsprodukte, so lässt sich wohl denken, dass dieselben bestimmte Funktionen haben, welcher Art sie auch sein mögen, sei es dass sie ihre Wirkung im Darmkanal entfalten, sei

es, dass sie, in die Blutbahn übergehend, in irgend einer Beziehung zur Zellenbildung im allgemeinen oder zur Knochenbildung stehen oder sonstige Allgemeinwirkung ausüben."

MEISSNER (1859) appears to have been the first to observe the peculiar behaviour of cow's casein towards pepsin. During peptic digestion of cow's casein, MEISSNER obtained a precipitate which when just formed had a jelly structure, and which did not dissolve during prolonged action of the enzyme. He called this pepsin-resistant substance "dyspepton". LUBAVIN (1871) showed that the dyspepton contained a substance with a high phosphorus content (4.6 %). He considered this substance to be a "nuclein".[1] WILLDENOW (1893) repeated LUBAVIN's work, and arrived at the same results. She obtained substances with properties similar to the nuclein described by LUBAVIN. For two different preparations she reports a phosphorus content of 3.85 and 4.66 % respectively. After removing the precipitate formed during the initial stages of digestion, CHITTENDEN and PAINTER (1885) isolated, from peptic digerates of cow's casein, a number of substances which were called caseoses. Some of these compounds, the protocaseoses, which could be separated from the digerate by means of sodium chloride and acetic acid, are reported to contain no phosphorus, but otherwise showed an elementary composition very similar to that of casein itself. CHITTENDEN and PAINTER submit two phosphorus analyses only, and in both cases they obtained a certain amount of phosphorus which they attributed to the presence of inorganic phosphate. It appears doubtful, however, whether their proteocaseoses really were free from organic phosphorus.

Among other investigations dealing with the course of the enzymatic breakdown of cow's casein may be mentioned those of THIERFELDER (1886) who, using a method devised by HOPPE-SEYLER (1883), involving precipitation with sodium chloride, hydrochloric acid and phosphotungstic acid, was able to isolate three different breakdown products from peptic digestion of casein. He called the three different products propepton I, propepton II and pepton respectively, and carried out carbon, hydrogen and nitrogen analyses. The name "pseudonuclein" for the insoluble product of the peptic digestion used below was suggested by HAMMARSTEN (1894). A little earlier (1891) KOSSEL had suggested the name "paranuclein". SEBELIEN (1895) pointed out that the pseudonuclein did not have a constant elementary composition, and that the amount formed during digestion decreases with increasing pepsin concentration.

Our knowledge of the peptic digestion of cow's casein was considerably enhanced by the work of SALKOWSKI and his collaborators

[1] At this time the name "nuclein" was applied to the digestion residue, insoluble in pepsin-hydrochloric acid mixture, which was obtained after the digestion of complicated compounds belonging to the protein class of substances and designated nucleoproteides by O. HAMMARSTEN (1894).

carried out between the years 1893 and 1901. Prior to SALKOWSKI's work, it had been assumed that the greater part of the phosphorus present in the casein went into the insoluble digestion products, cf. LUBAVIN (1871) and HOPPE-SEYLER (1893), and that the soluble part (the so-called albumoses) was free from phosphorus. SALKOWSKI (1893) showed, however, that by far the greater part of the phosphorus was contained in the soluble phase of the digestion mixture, and also that it was possible to run the enzymatic hydrolysis under such conditions that no insoluble pseudonuclein was formed. Similar results were obtained also by ALEXANDER (1898), but other workers, *e. g.* v. MORACZEWSKI (1895), SEBELIEN (1895) and WRÓBLEWSKI (1894), always obtained a small residue when digesting casein solutions with pepsin. SALKOWSKI and HAHN (1895) showed that the soluble phosphorus formed during digestion was present in organically bound form. This did not agree with the earlier work of v. SZONTAGH (1893) whose observations seemed to show that free phosphoric acid was formed during digestion. v. MORACZEWSKI (1895) was also of the opinion that under certain conditions inorganic phosphorus was formed during peptic digestion of cow's casein. The later workers in this field have fully confirmed the correctness of SALKOWSKI's view. SALKOWSKI (1901) undertook the isolation of the soluble organic phosphorus compound, *viz.* the "paranucleic acid". He isolated the latter substance in the form of iron salt, and found the following elementary composition: C 31.9, H 4.43, N 9.72, P 2.55, Fe 21.87 %. He also prepared iron-free "paranucleic acid" with the following composition C 42.96, H 7.09, N 13.55, P 4.05 %. SALKOWSKI also showed that the substance was unstable at alkaline reaction. Even the action of weak solutions of barium hydroxide led to the splitting-off of phosphoric acid in inorganic form.

REH (1908) prepared the uranium salt of "paranucleic acid" and obtained a product of constant composition in different preparations. On analysis, he obtained C 24.02, H 4.00, N 7.59, P 4.27, U 33.33 %. Calculated as free acid the substance contained 6.9 % of phosphorus as against a maximum value of 4.31 % reported by SALKOWSKI. REH showed that the paranucleic acid was, in fact, a polypeptide, and among the products of *acid* hydrolysis he reports the presence of the following amino acids: lysine, arginine, histidine, isoleucine, leucine, valine, glutamic acid, proline, aspartic acid. He suggested the name "Polypeptidphosphorsäure" instead of paranucleic acid.

In the year 1905 PLIMMER and BAYLISS investigated the separation of phosphorus from casein by the action of pepsin, trypsin, papain and alkali. In the case of peptic digestion they found that all of the phosphorus could not be precipitated by tannic acid, which SALKOWSKI's work had given them to expect, if all of it was in the form of paranucleic acid. After digestion for 149 days they found that the amount of phosphorus which could be precipitated by tannic acid was only about one

third of the total phosphorus. This did not agree with the findings of Salkowski. Plimmer and Bayliss attributed the difference to the longer time of digestion used in their experiments. They thought that the paranucleic acid was further hydrolyzed into a phosphorus-containing peptone which unlike paranucleic acid could not be precipitated by tannic acid. Plimmer and Bayliss also stated that only minute quantities of inorganic phosphoric acid are formed during digestion, a result earlier obtained by Salkowski, and later confirmed by Rimington and Kay (1926).

Robertson (1907) expressed the view that paranucleic acid was most probably a mixture of at least two different compounds, basing this opinion upon the very different content of phosphorus (0.88–6.86 %) reported by different authors.

Rimington and Kay (1926) investigated the action of bone phosphatase on the soluble phosphorus-containing complex from peptic digests, and found that the complex was stable towards the enzyme. The same was the case with paranuclein containing 1.4 % phosphorus.

Holter, Linderström-Lang and Funder (1932) carried out an extensive study of the peptic digestion of cow's casein. They were particularly interested in para- or pseudonuclein, for which they use the name phosphopeptone. Unfortunately, Rimington and Kay (1926) had already suggested this name for the organic phosphorus-containing compound which they had isolated from tryptic digests of casein. Holter, Linderström-Lang and Funder followed the viscosity change during peptic digestion of cow's casein. They found that, after an initial lowering, the viscosity increased and passed through a maximum. The increasing viscosity was attributed to the formation of "phosphopeptone". This phenomenon had also been observed by Waldschmidt-Leitz and Simons (1926). An initial lowering of viscosity is common in all enzymatic hydrolysis of proteins. Holter, Linderström-Lang and Funder also showed that the degree of cleavage, followed by means of titration in acetone, was independent of the changes in the viscosity curve *i. e.* the formation of the insoluble "phosphopeptone" did not appreciably change the course of the hydrolysis. These authors also studied the viscosity changes during peptic digestion of casein fractions prepared according to Linderström-Lang (1929) and containing varying amounts of phosphorus. The result was that the change in viscosity was less pronounced for casein fractions containing a small amount of phosphorus, and that no precipitate of "phosphopeptone" was obtained during the digestion of such fractions. The susceptibility of the different casein fractions towards pepsin was found to vary with the phosphorus content of the fractions, a high phosphorus content being offset by a high susceptibility towards the peptic cleavage. Holter, Linderström-Lang and Funder (1932) considered that their results could be explained on the assumption that cow's casein

is made up of two or three components with different phosphorus content. They express the view that the formation of "phosphopeptone" is an essential part of the peptic hydrolysis of casein. They also think that their "phosphopeptone" is an individual compound, basing this opinion upon the finding that "phosphopeptone" prepared from different casein fractions, subjected to approximately the same degree of enzymatic cleavage, had the same P/N ratio (0.11–0.12, corresponding to an atomic N P ratio of 20–18). Precipitated "phosphopeptone" present as a solid phase in the digestion mixture is further hydrolyzed by the enzyme, indicated by a rising P/N ratio on prolonged hydrolysis.

Pseudonuclein was prepared by Damodaran and Ramachandran (1941) as an intermediate stage in the preparation of trypsin-resistant phosphopeptone. They digested a suspension of casein in dilute sulphuric acid at pH 2 until a constant value of amino nitrogen (usually about 20 %) was reached. This happened after about a week's digestion. No inorganic phosphorus was formed during the peptic digestion. The residue of pseudonuclein gave, after drying, the following analytical figures: P, 2 %, N, 15.2–15.6 %; 12 % of the nitrogen was in the form of amino nitrogen determined by the method of Van Slyke. From about one kilogram of casein they obtained a yield of 200 g of pseudonuclein containing about 20 % of the nitrogen and 50—60 % of the phosphorus in the original casein.

Summarizing the earlier work on peptic breakdown of cow's casein, it may thus be said to be established that only traces of inorganic phosphorus are split off and that compounds containing organically bound phosphorus are formed. Depending on the mode of isolation these compounds show very different composition, but they always contain appreciably more phosphorus pro nitrogen than the casein itself. It is also evident that the formation of a gel in the early stages of digestion, and a precipitate of pseudonuclein later on, are characteristic features of cow's casein when digested with pepsin or gastric juice.

There seems to have been considerable interest in the peptic digestion of human casein during the later part of the nineteenth century. The experiments were of a qualitative nature, however, and the preparative methods used were often such as to suggest that the casein employed was not free from other milk proteins. Main interest has centred round the comparison of the course of the peptic breakdown in the case of cow's and human casein respectively. Simon (1840) enclosed equal-sized lumps of cow's and human casein respectively in parts of an infant's stomach, and noted that the lump of human casein was digested in a shorter time. Biedert (1869, 1874) was also of the opinion that human casein was more easily digested than cow's casein, and that the two caseins were chemically rather different. Langgaard (1875) held the same view. This author also digested casein from mare's milk, and found it to be nearly as easily digested as human casein. The method of these

early authors was to compare the insoluble residue of the casein digerates. When this residue was large, the degree of digestion was thought to be low and, conversely, when no residue was formed, the digestion was said to have been complete. In the light of later investigations it may be said that the difference found between human and cow's casein is due to the property of cow's casein solutions to form a precipitate (of pseudonuclein) during the peptic digestion, which is not the case with human casein.

Dogiel (1885) appears to have been the first to study this question. He reports that the peptic digestion of human casein follows the same course as that of cow's casein, with the formation of "nuclein", indicated by the formation of a very weak gel ("kann endlich schwach gelatinös werden"). Dogiel carried out comparative digestion experiments without noticing any differences in the behavior of the two caseins. He found that "die beiden Kaseine bei der Pepsinverdauung dieselben Peptone in gleicher Menge liefern". v. Szontagh (1893), however, did not observe any formation of pseudonuclein during the peptic digestion of human casein. Wróblewski (1894) a little later confirmed this result. He also found no pseudonuclein formed during digestion of human casein with extracts of the gastric mucosa of human infants. After Wróblewski, v. Moraczewski (1895) confirmed the absence of pseudonuclein formation in peptic digests of human casein. Wróblewski also digested identical solutions of cow's casein with pepsin from swine and human infants respectively. He observed in both cases the formation of a precipitate of nuclein, which on prolonged digestion was dissolved only to a small extent. No quantitative determination of the degree of hydrolysis was performed, however. It must be recalled that, at the time Wróblewski's investigation was carried out, no quantitative methods had been developed. Wróblewski states that cow's casein and human casein are chemically different, but says nothing concerning a difference in digestibility between the two caseins. It is still not quite clear to what extent casein is broken down during physiological digestion in the infant's stomach. Comparative digestion experiments using cow's and human casein respectively as substrates, and gastric juice from infants as the source of enzyme, seem previously to have been carried out only by Somló and Szirmay (1924), who in the course of an investigation dealing with the adaptation of digestive enzymes, describe two experiments in which they digested solid cow's and human casein respectively with the same gastric juice. The degree of digestion was determined refractometrically. In one experiment in which gastric juice from an infant five weeks old was used, the cow's casein showed a considerably higher degree of digestion than did the human casein. In the second experiment the gastric juice was taken from an infant nine weeks old, and in this case the same relative increase in the refraction was ob-

tained for both caseins. Apart from these experiments, which were performed for other purposes, no one seems to have published any comparative experiments into the breakdown of human and cow's casein in the stomach of infants or *in vitro* with gastric juice from infants. On the other hand, numerous digestion experiments have been performed with diluted and undiluted cow's milk and with human milk. From such experiments conclusions have been drawn regarding the digestibility of the proteins of milk under different conditions. It is clear, however, that experiments of this kind do not allow of any definite conclusions with regard to the behaviour of the different proteins present in milk in the digestion process. Digestion experiments on whole milk are therefore of no immediate interest in our present problems. We mention a few, however. Simon (1838) reports that mucosa from infant's stomach causes a more complete coagulation of human milk than that from calf's fourth stomach. Biedert (1869) found that, in dog's stomach, human milk was more easily digested than cow's milk. Van Bueren (cited from Biedert 1869), on the other hand, reports that in calf's stomach human milk is more slowly digested than is cow's milk. For a detailed discussion of the early literature the reader is referred to the work of Wróblewski (1894).

Some of the earlier authors denied the possibility of a proteolysis in the infant's stomach. Davidsohn (1911, 1912, 1913) was of the opinion that no appreciable peptic hydrolysis could occur on account of the low acidity which he found in gastric content from infants. Salge (1912, 1913) took the same view. Rosenbaum and Spiegel (1925) digested diluted and undiluted cow's milk and human milk *in vivo* for 1 $^1/_2$ hour. After this time the digestion mixture was taken up and digestion allowed to proceed in a thermostat. The increase in the nitrogen content in the filtrates after precipitating with trichloroacetic acid was determined, and taken as a measure of the proteolysis. They arrived at the conclusion that proteolysis occurred when undiluted or twice diluted cow's milk was used, but not when human milk or more diluted cow's milk was employed. The authors considered that the protein content of the milk was a deciding factor. They also observed that digestion took place even though the hydrogen ion concentration of the digerates was far from the optimal pH for peptic hydrolysis. The remarkable point is that no digestion of the human milk was observed. Budde (1929) points out that, in the experiments just referred to, one cannot altogether rule out the possibility that proteolysis of the human milk had taken place in the stomach; that this digestion had come to an end after 1 $^1/_2$ hour, and that therefore no increase in filtrate nitrogen was observed afterwards *in vitro*. Hess (1927) arrived at the same results as Rosenbaum and Spiegel (1925) and was also of the opinion that cow's but not human milk protein was digested.

Budde (1929) has determined the degree of digestion of human milk

and diluted cow's milk after digestion for one hour in the stomach of infants. Digestion was followed by titration in alcoholic solution according to WILLSTÄTTER *et al.* (1926). The pH of samples taken at intervals varied between 3.0 and 6.4, and human milk showed a degree of digestion about one-third that of twice-diluted cow's milk. BUDDE ascribes this difference to the relatively higher content of albumin in human milk. In separate experiments she has shown that lactalbumin is considerably more resistant towards swine pepsin than is cow's casein. A 3 % solution of cow's casein was compared with a 3 % solution of albumin (lactalbumin or eggalbumin). In the pH range 2—6 the degree of cleavage for the albumin solutions was considerably lower than for the casein solution.[1] At optimal pH the cleavage of the lactalbumin was about one-third that of cow's casein. Similar results were obtained in *in vitro* experiments, when undiluted human milk and thrice-diluted cow's milk was digested with gastric juice from infants. Maximum hydrolytic cleavage occurred between pH 2 and 3 but a distinct action was still found at pH 5. Although the main results of BUDDE's experiments are probably correct, some criticism can be directed against her experimental technique. In most of the experiments on milk and casein "solutions" the casein has been present in the form of a precipitate in the digestion mixture. In the comparative experiments with albumins the protein has been in solution, however. Unless there is evidence to the contrary, it cannot be taken for granted that a strict comparison is possible between substrates of such different physical states. No experiments comparing the peptic digestibility of human and cow's casein were made. The experiments carried out in the course of the present work indicate that the difference observed by BUDDE in the digestibility of the two sorts of milk may, at least in part, be due to differences between the caseins.

WILLSTÄTTER and BAMANN demonstrated 1928 that gastric mucosa from swine contains a proteolytic enzyme active at weakly acidic reaction with an optimum at pH 3.5—4. They suggested the name cathepsin for this enzyme and expressed the opinion that the enzyme and also an enzyme of the erepsin type originate from the leucocytes in the gastric mucosa. With regard to the human infant, however, FREUDENBERG in a paper published in 1932 denies the existence of a proteolytic enzyme of the cathepsin type in the gastric juice, basing this opinion on experiments carried out by himself and by BUDDE (1929). FREUDENBERG states that he has always found a continually decreasing proteolytic activity of gastric juice on changing the reaction towards the alkaline side (pH 1.8—pH 5.0), and that he has never observed a second maximum.

[1] A similar difference in digestibility between casein and serumalbumin (or serumglobulin) was described by ABDERHALDEN and KRÖNER (1928).

In more recent publications FREUDENBERG *et al.* (1940, 1941[1]) interpret these results in a different manner. In a new series of experiments they found that cow's milk and solutions of egg albumin are digested to a considerable degree at pH 4.7 by gastric juice from infants under two years of age. The degree of digestion was determined in a manner similar to that used by ROSENBAUM and SPIEGEL. FREUDENBERG considers that the digestion at pH 4.7 must be caused by an enzyme of the cathepsin type. FREUDENBERG (1941) also prepared cathepsin from gastric juice from infants practically free from pepsin by precipitating the juice with 34 % sodium chloride and heating it to a temperature of 40° C for 15 minutes. The optimal reaction for the cathepsin preparations thus obtained was about pH 4.5. FREUDENBERG assumes that no peptic action is possible at this pH (cf. NORTHROP 1923). The optimal catheptic action took place at a temperature of 60° C and the enzyme was activated by hydrogen sulphide and hydrogen cyanide.

SEBELIEN (1895) was the first to investigate the behaviour of cow's casein towards pancreatic extracts. He found that digestion of the casein with extract from ox pancreas led to a nearly complete breakdown of the protein, and that the total phosphorus of the casein went into soluble products. BIFFI (1898) showed that the phosphorus of the digerates was present partly in inorganic form (about 27 % of the total phosphorus could be precipitated by magnesia mixture) and partly in organic form. The amount of the latter decreased on continued digestion, and when larger amounts of pancreatic extracts were used. Both SEBELIEN and BIFFI pointed out that a small amount of indigestible residue containing phosphorus was precipitated on acidifying their digerates. They were of the opinion that this phosphorus-containing substance was derived from the pancreatic extract and that it had no relation to the casein, basing this opinion on the fact that the amount of the residue increased when larger amounts of pancreatic extract were used.

In 1905 PLIMMER and BAYLISS investigated the digestion of cow's casein with "pancreatin". They followed the hydrolysis by precipitating undigested protein with tannic acid and determining the amount of "soluble P_2O_5" in the filtrate. A small quantity of phosphorus always remained in the tannic acid precipitate. The authors consider, however, that this phosphorus is derived from an impurity in the casein or from the pancreatic extract. PLIMMER and BAYLISS also investigated the nature of the "soluble P_2O_5". In a digestion experiment which had been continued for nearly two months, all the phosphorus was present in a soluble form, but only about 35 % was inorganic. About 65 % of the phosphorus was thus in organic combination,

[1] cf. also FREUDENBERG and BUCHS (1940) and BUCHS (1940).

and about half of this phosphorus could be converted into inorganic form by the action of 1 % NaOH. The organically bound part of the phosphorus could be completely precipitated with ferric ammonium sulphate in the same manner as the paranucleic acid of SALKOWSKI (1901). PLIMMER (1913) found that all of the phosphorus in the phosphorus-containing organic substance of the digerates could be split off by alkali and by extracts from the intestinal mucosa of cats or dogs. PLIMMER and BAYLISS have thus shown the presence in the casein digerates of a compound containing organically bound phosphorus, resistant to pancreatin but sensitive to the action of alkali and extract from intestinal mucosa. Neither PLIMMER and BAYLISS (1905) nor PLIMMER (1913) made any attempts to isolate the phosphorus-containing organic substance. RIMINGTON and KAY (1926) digested casein with pancreatic extracts and found that, provided the concentration of enzyme was sufficient, all organically bound phosphorus was converted into inorganic form. According to RIMINGTON and KAY these experiments modify the views of the previous authors by showing that the organic phosphorus-containing compound (called phosphopeptone) formed during the tryptic digestion of casein is not quite stable towards prolonged action of high concentrations of the enzyme. The authors themselves have shown, however, that extracts from intestinal mucosa containing phosphatase bring about a rapid hydrolysis of the phosphopeptone. To the present author it appears obvious, therefore, that the complete hydrolysis obtained with pancreatic extract activated with enterokinase is due to the combined action of proteolytic enzymes and phosphatases. The pH of the digestions was about 8.5 and this is nearly the optimum pH for the action of intestinal phosphatases. RIMINGTON and KAY do not appear to have carried out any control experiments with phosphatase-free trypsin. The same authors have, however, isolated the phosphopeptone from the digerates and found it to contain over 60 % of the phosphorus from the original casein. Their phosphopeptone was acidic in nature and formed insoluble salts with a series of heavy metals but a barium salt freely soluble in water. It contained 10 % nitrogen and 4 % phosphorus. After RIMINGTON and KAY several others have prepared and described phosphopeptone under different names (see chapter IV).

AUTHOR'S EXPERIMENTS

The peptic digestion of cow's and human casein

As mentioned above, several authors have found qualitative differences between the courses of the peptic digestion of human and cow's casein respectively. With one exception the investigations cited appear to show that no pseudonuclein is formed during peptic digestion of human

casein. In the case of cow's casein, it has been shown by the work of HOLTER, LINDERSTRÖM-LANG and FUNDER (1932) that the precipitation of pseudonuclein is preceded by a strong increase in the viscosity of the solution. Casein fractions with low phosphorus content which give a weak or no precipitate of pseudonuclein also give a much smaller increase in the viscosity during digestion. As the increase in viscosity during peptic digestion is apparently a sensitive response to the formation of pseudonuclein, the viscosity changes during peptic digestion of human casein have been studied. No quantitative comparisons between the peptic digestibility of human and cow's casein appear to have been carried out previously. The present author has therefore made such experiments using identical experimental conditions in the digestion of both caseins with commercial and crystalline swine pepsin, as well as with gastric juice from infants (in one experiment gastric juice from an adult was used).

To investigate how the activity of pepsin and gastric juice is altered when the hydrogen ion concentration is changed from the optimum at about pH 2 towards the alkaline side, some digestion experiments between pH 1.5 and 3.5 have been carried out. As already mentioned, when the reaction is more alkaline than pH 3.5—4 a precipitate is formed in the casein solutions, which renders the formol titrations uncertain. For this reason experiments at more alkaline reactions have not been performed. In these experiments, too, commercial and crystalline pepsin, as well as gastric juice from infants, were used.

The peptic breakdown of the caseins *in vitro* and *in vivo* has also been followed electrophoretically.

Preparation of casein solutions: 2.5 g of human and cow's casein (water content 7—9 %) respectively were thoroughly ground with a small amount of water (25 ml) to a homogeneous mixture in a glass mortar, and the mixture left overnight in the refrigerator. Next morning water was added to make the volume about 70 ml and then 0.1 N sodium hydroxide (usually 10—12.5 ml) added until the pH of the resulting mixture was 6.5—7.0. A little toluene was added and the mixture placed in the shaking machine for two hours. After this time the casein was completely dissolved. The solution was now added in small portions to a volume of 12.5 ml (in some experiments 10 ml) of 0.3 N hydrochloric acid. A further quantity of 0.1 N sodium hydroxide was then added to make the total volume of added sodium hydroxide equal to the volume of 0.3 N hydrochloric acid used. Water was finally added to bring the volume up to 100 ml. In some cases when the water content of the casein preparations used was unknown, the nitrogen content of the solution was determined by KJELDAHL'S method and water added to the solution of the higher nitrogen value to make the protein concentrations of the two solutions equal.

The pH of casein solutions prepared in the manner just described

was usually about 2.1—2.2, independent of the casein preparations used. Minor pH differences between the two casein solutions used in a digestion experiment were corrected by adding a few drops of 0.3 N sodium hydroxide or hydrochloric acid. When a solution more acid or more alkaline than pH 2.2 was wanted, the pH was adjusted by adding 0.1 N sodium hydroxide or 0.3 N hydrochloric acid.

pH Measurements: All determinations of the hydrogen ion concentration were performed with the glass electrode. The potentials were measured with a Leed's & Northrup type K 2 potentiometer and a COMPTON electrometer (short period pattern, Cambridge Instrument Co. Ltd.). All measurements were carried out at $20 \pm 0.1^{\circ}$ C in an apparatus for electrometric titrations designed by STENHAGEN (1938). A calomel electrode with saturated potassium chloride was used as reference electrode. The glass electrodes were of the MACINNES type (Corning glass No. 015) or of the bulb type (the latter were made by Radiometer Ltd., Copenhagen). The electrodes were calibrated with the aid of three different buffers, prepared according to CLARK (1928), using the pH values given in his tables. In most cases a phosphate buffer of pH 6.81, a sodium acetate-acetic acid buffer of pH 4.62 and a sodium phtalate-hydrochloric acid buffer of pH 2.40 were used. The glass electrode was calibrated before each experiment. The reproducibility of the measurements was always better than 0.05 pH units. In some of the later experiments a potentiometer of the Beckman type (Industrial Model) was used. In such experiments the pH figures are given to the first decimal place only.

Digestion procedure: Solutions of human and of cow's casein containing the same amount of protein and of the same hydrogen ion concentration (within 0.05 pH units) were left in a water thermostat at a temperature of 37° C (in some cases 30° C) for one hour. The freshly prepared enzyme solution, warmed in a similar manner to the same temperature, was then added. In most experiments a 2 % solution of a pepsin preparation (Parke, Davis & Co. 1/3000) was used. The pepsin concentration in the digerates was kept at 0.05 % (*i. e.* 0.05 g of the commercial pepsin per 100 ml digerate). In one series of experiments crystalline pepsin prepared according to the method of NORTHROP (1930) as modified by PHILPOT (1935) was used.[1] In this case a pepsin concentration corresponding to 8 mg of enzyme protein per 100 ml digerate was used.

Viscosity measurements: OSTWALD type viscosimeters, having a volume of about one ml between marks, were used. Immediately before or after each experiment the emptying time of the viscosimeter at the temperature of the experiment was determined, using doubly distilled water. The emptying time was measured to the nearest 0.2 sec. The

[1] The author is indebted to Dr. GUNNAR ÅGREN for this enzyme preparation.

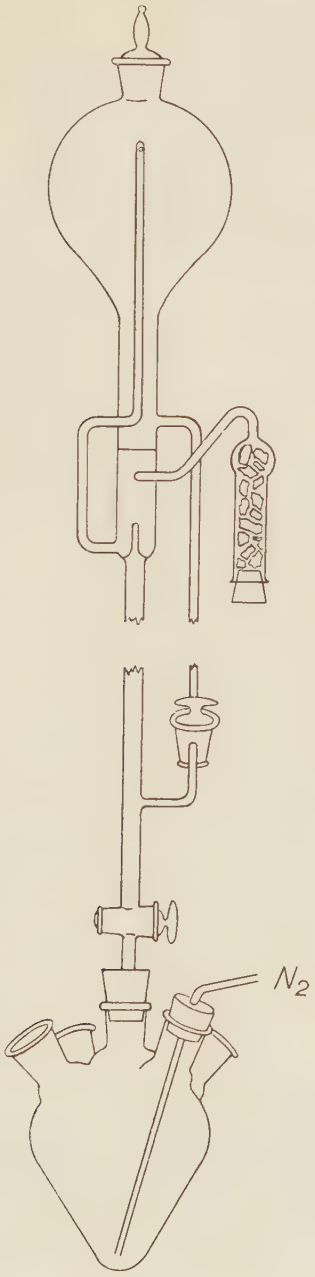

Fig. 12.

Digestion experiment No. 1 (cf. Fig. 13).

Casein solutions: 2.5 g of cow's casein V and human casein 36 respectively, 10 ml of 0.1 N sodium hydroxide, 10 ml of 0.3 N hydrochloric acid, and distilled water to bring the volume up to 100 ml.
Enzyme: pepsin (Parke, Davis & Co. 1 3000) 0.05 %.
Hydrogen ion concentration: solution of cow's casein pH 2.18.
solution of human casein pH 2.23.
Temperature: 30° C. *Water values of viscosimeters:* 11.0 and 11.6 seconds respectively.

Cow's casein					Human casein				
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at zero time	Relative viscosity	Difference from value at zero time	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at zero time	Relative viscosity	Difference from value at zero time
Before adding enzyme	5.32	0.00	1.54	0.00	Before adding enzyme	3.96	0.00	1.78	0.00
3			1.36	−0.18	2			1.68	−0.10
8			1.27	−0.27	9			1.64	−0.14
13			1.28	−0.26	13	4.05	0.09		
15	5.56	0.24			19			1.56	−0.22
16			1.32	−0.22	21			1.56	−0.22
23			1.32	−0.22	22			1.56	−0.22
25	5.66	0.34			29	4.10	0.14		
27			1.36	−0.18	32			1.47	−0.31
32			1.45	−0.09	35			1.47	−0.31
34	5.72	0.40			42	4.16	0.20		
36			1.64	+0.10	44			1.38	−0.40
40			1.77	+0.23	54	4.22	0.26		
42	5.78	0.46			61			1.38	−0.40
43			1.91	+0.37	81	4.23	0.27		
46			2.01	+0.47	85			1.30	−0.30
50			2.45	+0.91	123			1.30	−0.30
52	5.80	0.48							
55			3.46	+1.92					
60			5.82	+4.28					
63	5.84	0.52							
65			gel formation						

relative viscosity was obtained by dividing the measured emptying time by the water value of the viscosimeter. Between readings the viscosimeter was kept closed to prevent evaporation.

Formol titrations[1]: The titration apparatus is shown in Fig. 12. A titration vessel of the type described by STENHAGEN (1938) is fitted

[1] Mainly as described by NORTHROP (1925).

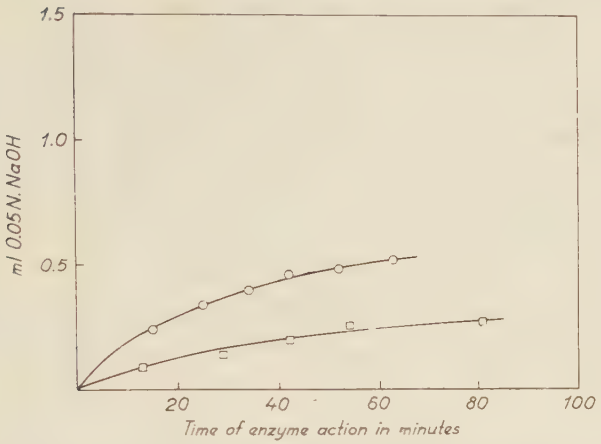

Fig. 13. Hydrolysis of cow's (O) and human (□) casein with commercial pepsin. Experimental data are given in digestion experiment No. 1.

with a Derona burette of 3 ml volume and with an inlet tube for purified nitrogen. In order to prevent the precipitation of casein from the acid solutions during titration it was necessary to proceed as follows. The amount of 0.05 N sodium hydroxide needed to neutralize 5 ml of the casein solution before addition of enzyme was determined in preliminary experiments. Slightly less (about 10 %) than the amount thus found was let out from the burette into the titration vessel previously filled with nitrogen. 1 ml of neutralized 40 % formaldehyde solution, two drops of secondary octyl alcohol and two drops of a 1 % solution of phenolphthalein were then added. Exactly 5 ml of the digestion mixture was then transferred to the titration vessel. The solution in the vessel was stirred by a steady stream of nitrogen from the inlet tube, and titration continued with 0.05 N sodium hydroxide to a faint pink colour. In some experiments titration had to be made on 15 ml of digerate to enable small hydrolytic cleavages to be followed. In this case 3 ml of the formaldehyde solution was used.

In order to obtain values to which the changes in viscosity and the formol titre respectively could be referred, viscosity and formol titre were initially determined on the casein solutions before addition of the enzyme. Control experiments using heat-inactivated (70° C during 10 minutes) enzyme solutions showed that no significant differences in the above determinations were caused by addition of the enzyme. During the first 2 minutes of digestion the titration values differed only very slightly from the zero time values obtained in the above manner (cf. digestion experiments Nos. 4 and 5). In later experiments the one- or two-minute values were therefore used as the reference points

Digestion experiment No. 2 (cf. Figs. 14 and 15).

Casein solutions: about 2.5 g of cow's casein XVIII and human casein 38 respectively (water content of both caseins unknown), 10 ml of 0.1 N sodium hydroxide, 10 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml. After nitrogen analysis, the solutions were diluted to a protein concentration of 1.7 % in both cases. To 100 ml of the cow's casein solution was added 1 ml of 0.3 N hydrochloric acid and to 100 ml of the solution of human casein were added 0.6 ml of 0.3 N hydrochloric acid and 0.4 ml of water.

Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.

Hydrogen ion concentration: solution of cow's casein pH 1.93,
solution of human casein pH 1.94.

Temperature: 37° C. *Water value of viscosimeter:* 9.2 sec.

Cow's casein					Human casein				
1	2	3	4	5	1	2	3	4	5
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at one minute	Relative viscosity	Difference from value at one minute	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at one minute	Relative viscosity	Difference from value at one minute
1	5.20	0.00	1.30	0.00	1	4.24	0.00	1.63	0.00
3			1.30	0.00	3			1.63	0.00
5			1.25	−0.05	4			1.61	−0.02
6	5.31	0.11	1.25	−0.05	5	4.28	0.04	1.61	−0.02
7			1.25	−0.05	8			1.54	−0.09
8			1.25	−0.05	10			1.56	−0.07
10			1.25	−0.05	12			1.57	−0.06
12	5.36	0.16	1.25	−0.05	13	4.33	0.09	1.56	−0.07
15			1.25	−0.05	15			1.54	−0.09
17			1.25	−0.05	17			1.54	−0.09
18			1.30	0.00	20	4.42	0.18		
19			1.30	0.00	21			1.54	−0.09
20	5.39	0.19			24			1.54	−0.09
21			1.30	0.00	26			1.52	−0.11
22			1.32	+0.02	28			1.52	−0.11
24			1.36	+0.06	29			1.52	−0.11
26			1.41	+0.11	30	4.47	0.23		
28			1.46	+0.16	32			1.52	−0.11
29			1.52	+0.22	35			1.52	−0.11
30	5.55	0.35	1.52	+0.22	40	4.50	0.26	1.52	−0.11
32			1.54	+0.24	46			1.52	−0.11
33			1.58	+0.28	50			1.51	−0.12
35			1.64	+0.34	54			1.50	−0.13
37			1.68	+0.38	55	4.56	0.32		
39			1.70	+0.40	60			1.47	−0.16
40	5.60	0.40			70	4.56	0.32		
41			1.74	+0.44	73			1.41	−0.22
43			1.76	+0.46	80			1.41	−0.22
46			1.80	+0.50	85	4.54	0.30	1.40	−0.23

1	2	3	4	5	1	2	3	4	5
48			1.86	0.56	100	4.60	0.36	1.41	0.22
50			1.90	0.60	115			1.41	0.22
52			1.96	0.66	120	4.54	0.30		
53			1.98	0.68					
55	5.71	0.51	2.05	0.75					
58			2.10	0.80					
60			2.16	0.86					
64			2.40	1.10					
71			2.60	1.30					
78			3.00	2.60					
85	5.80	0.60	4.25	2.05					
90			gel formation						
100	5.80	0.60							
130	5.80	0.60							

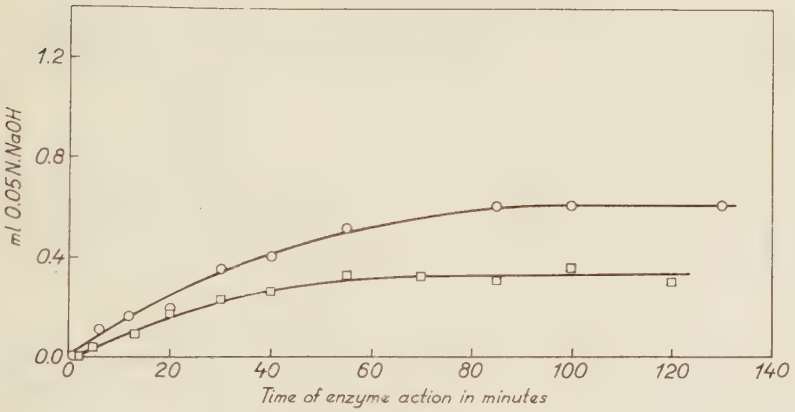

Fig. 14. Hydrolysis of cow's (○) and human (□) casein with commercial pepsin. Experimental data are given in digestion experiment No. 2.

for the formol titrations. As it was not always possible to carry out the first viscosity determination two minutes after the start of digestion, a value obtained at a later time had sometimes to be used as reference point for the viscosity measurements. The end points of the titrations are reproducible within 0.04 ml 0.05 N sodium hydroxide.

Gastric juice was obtained partly from healthy infants and partly from infants recovering from pyloric stenosis. The age of the infants varied between one and six months. The gastric content was drawn both before the first meal in the morning (infants suffering from pyloric stenosis give considerable amounts of active juice), and two to three

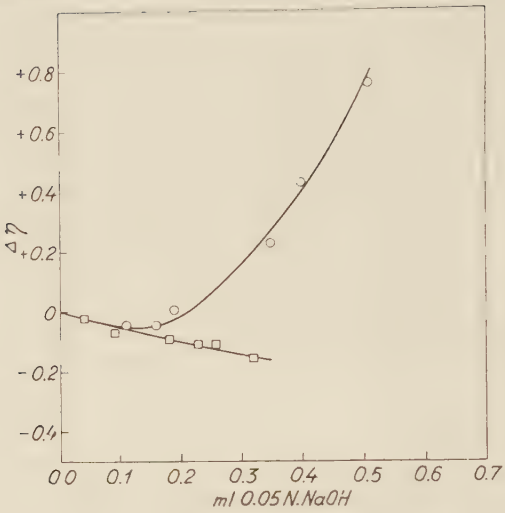

Fig. 15. Change in relative viscosity ($\Delta\,\eta$) during hydrolysis of cow's (O) and human (□) casein with commercial pepsin. Experimental data are given in digestion experiment No. 2.

Digestion experiment No. 3.

Casein solutions: 2.5 g of cow's casein X and human casein 49 respectively, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid. A further portion of 2 ml 0.1 N sodium hydroxide was added to both solutions before filling up with water to 100 ml.

Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.

Hydrogen ion concentration: solution of cow's casein pH 2.99,
solution of human casein pH 2.99.

Temperature: 37° C.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	5.04	0.00	2	5.14	0.00
16	5.18	0.14	12	5.18	0.04
25	5.24	0.20	21	5.20	0.06
32	5.26	0.22	28	5.22	0.08
40	5.28	0.24	36	5.24	0.10
48	5.32	0.28	45	5.25	0.11
63	5.34	0.30	56	5.25	0.11
81	5.42	0.38	75	5.30	0.16
96	5.44	0.40	90	5.30	0.16
115	5.46	0.42	106	5.30	0.16
155	5.44	0.40	150	5.30	0.16

Digestion experiment No. 4.

Casein solutions: 2.5 g of cow's casein X and human casein 39 respectively, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.03 N hydrochloric acid. A further portion of 5 ml 0.1 N sodium hydroxide was added to both solutions before filling up with water to 100 ml.
Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.
Hydrogen ion concentration: solution of cow's casein pH 3.33, solution of human casein pH 3.37.
Temperature: 37° C.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
Before adding enzyme	4.59		Before adding enzyme	4.80	
2	4.62	0.00	2	4.82	0.00
15	4.69	0.07	12	4.86	0.04
26	4.72	0.10	20	4.86	0.04
34	4.78	0.16	30	4.86	0.04
43	4.82	0.20	40	4.88	0.06
55	4.80	0.18	50	4.94	0.12
75	4.90	0.28	61	4.94	0.12
90	5.00	0.38	79	4.99	0.17
110	4.98	0.36	97	5.00	0.18
126	5.00	0.38	117	5.01	0.19
152	5.00	0.38	150	5.02	0.20

hours after a breast meal. The mixture of digested milk and gastric juice thus obtained was centrifuged and filtered through glass wool. The resulting liquid will be called "gastric juice". The most active samples were picked out and mixed. In one case gastric juice from an adult healthy person was used. It was obtained after injection of histamine.

In order to find out whether the difference in digestibility found in experiments Nos. 1—8 between the two caseins when using commercial pepsin at different pH (1.9—4.0) and gastric juice about pH 2 also existed when crystalline pepsin or gastric juice were used at varying hydrogen ion concentration the following experiments have been carried out. These experiments also allow a coarse comparison of the pH-optima of the peptic digestion of the two caseins with crystalline or commercial pepsin and with gastric juice respectively.

The same casein solution was used at different hydrogen ion concentrations, the latter being adjusted by adding an appropriate amount

Digestion experiment No. 5.

Casein solutions: 3.75 g of cow's casein X and human casein 39 respectively; 18.75 ml of 0.1 N sodium hydroxide, 18.75 ml of 0.3 N hydrochloric acid. Before filling up with water to 150 ml 30 ml of 0.1 N sodium hydroxide were added to the solution of cow's casein and 22.5 ml of 0.1 N sodium hydroxide to the solution of human casein.

Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.

Hydrogen ion concentration: solution of cow's casein pH 4.04, solution of human casein pH 4.02.

Temperature: 37° C.

Water values of viscosimeters: 19.5 and 18.0 seconds respectively.

Cow's casein					Human casein				
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Relative viscosity	Difference from value at ten minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Relative viscosity	Difference from value at five minutes
Before adding enzyme	2.98				Before adding enzyme	3.80			
2	3.00	0.00			2	3.82	0.00		
10			1.43	0.00	5			1.30	0.00
15	3.07	0.07			10	3.82	0.00	1.30	0.00
18			1.43	0.00	18	3.85	0.03		
33	3.12	0.12			33	3.89	0.07		
39			1.82	+0.39	38			1.28	−0.02
45	3.17	0.17			50	3.92	0.10		
55			3.10	+1.67	55			1.30	0.00
58			3.50	+2.07	67	3.94	0.12		
60	3.23	0.23			75			1.30	0.00
70			4.05	+2.62	93	3.95	0.13		
			gel		110			1.30	0.00
90			forma-		114	3.96	0.14		
			tion		139	3.96	0.14		
125	3.29	0.29			147			1.30	0.00
					170	3.97	0.15		
					175			1.34	+0.04
					205	3.97	0.15		
					212	3.98	0.16		
					215			1.31	0.01
					240	4.00	0.18		
					245	4.00	0.18		

Digestion experiment No. 6.

Casein solutions: 2.5 g of cow's casein V and human casein 38 respectively, 10 ml of 0.1 N sodium hydroxide, 10 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.

Enzyme: gastric juice from an adult person. 5 ml of the gastric juice was added to 80 ml of cow's casein solution, and 2.5 ml to 40 ml of the human casein solution.

Hydrogen ion concentration: solution of cow's casein pH 2.20,
solution of human casein pH 2.20.

Temperature: 30° C.

Water values of viscosimeters: 11.5 and 10.5 seconds respectively. Unfortunately the first formol titrations of this experiment were lost by accident. It has therefore been necessary to take the 12-minute values as zero values for the digestions.

Cow's casein					Human casein				
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at twelve minutes	Relative viscosity	Difference from value at six minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at twelve minutes	Relative viscosity	Difference from value at four minutes
6			1.30	0.00	4			1.24	0.00
12	5.48	0.00			12	4.53	0.00		
16			1.30	0.00	14			1.24	0.00
19	5.55	0.07			23.5	4.53	0.00		
28			1.30	0.00	27			1.24	0.00
30	5.60	0.12			33	4.54	0.01		
37			1.30	0.00	35			1.24	0 00
47			1.56	+0.26	46			1.24	0.00
51			1.62	+0.32	50			1.19	−0.05
53			1.64	+0.34	54			1.22	−0.02
57	5.76	0.28			60			1.24	0.00
62			2.09	+0.79	64	4.66	0.13		
65.5			2.52	+1.22	66			1.19	−0.05
74			3.70	+2.40	80			1.24	0.00
75	5.78	0.30			84	4.66	0.13		
82			4.60	+3.30	91			1.24	0.00
90			5.02	+3.72	105	4.64	0.11		
95	5.80	0 32	incipient		125			1.24	0.00
			precipitation		150			1.24	0.00
101			4.78	+3.48	160	4.66	0.13		
127	5.86	0.38							
155	5.86	0.38							

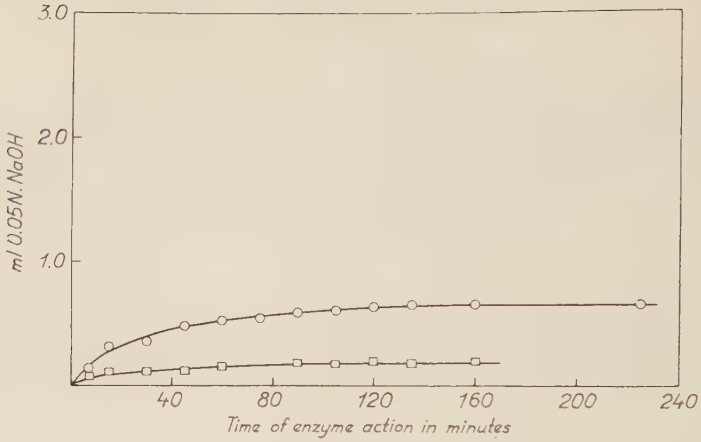

Fig. 16. Hydrolysis of cow's (O) and human (□) casein with gastric juice from infants. Experimental data are given in digestion experiment No. 7.

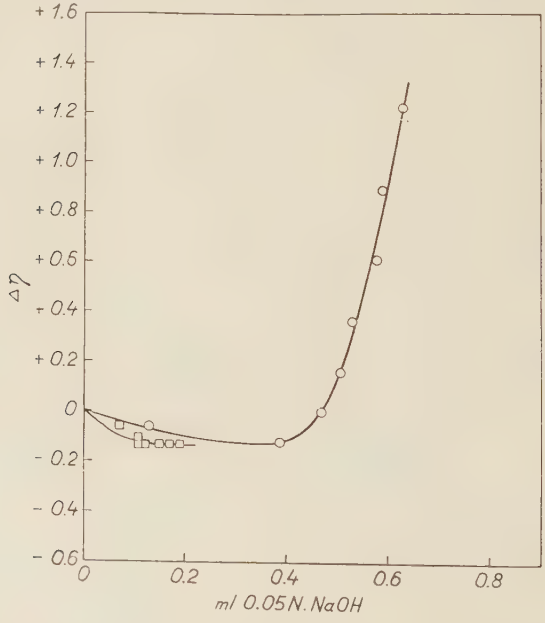

Fig. 17. Change in relative viscosity ($\Delta\ \eta$) during hydrolysis of cow's (O) and human (□) casein with gastric juice from infants. Experimental data are given in digestion experiment No. 7.

Digestion experiment No. 7 (cf. Figs. 16 and 17).

Casein solutions: 2.5 g of cow's casein XVIII and human casein 19, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.

Enzyme: mixed gastric juice from infants. 5 ml of the gastric juice was added to 80 ml of casein solution.

Hydrogen ion concentration: solution of cow's casein pH 2.02.
solution of human casein pH 2.00.

Temperature: 37° C.

Water values of viscosimeters: 12.0 and 18.0 seconds respectively.

Cow's casein					Human casein				
1	2	3	4	5	1	2	3	4	5
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at one minute	Relative viscosity	Difference from value at one minute	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at one minute	Relative viscosity	Difference from value at one minute
1	6.05	0.00	1.21	0.00	1	5.01	0.00	1.18	0.00
2			1.19	− 0.02	2			1.15	− 0.03
3			1.16	− 0.05	7	5.08	0.07	1.12	− 0.06
4			1.13	− 0.08	11			1.10	− 0 08
5			1.12	− 0.09	13			1.10	− 0.08
7	6.18	0.13	1.15	0.06	15	5.12	0.11	1.07	− 0.11
8			1.12	− 0.09	17			1.08	− 0.10
10			1.12	− 0.09	20			1.07	− 0.11
12			1.10	− 0.11	25			1.10	− 0.08
14			1.10	− 0.11	30	5.12	0.11	1.05	− 0.13
15	6.36	0.31			35			1.05	− 0.13
17			1.08	− 0.13	40			1.05	− 0.13
21			1.08	− 0.13	45	5.13	0.12	1.05	− 0.13
25			1.10	− 0.11	50			1.05	− 0.13
30	6.44	0.39	1.08	0.13	55			1.06	0.12
35			1.10	− 0.11	60	5.16	0.15	1.05	− 0.13
40			1.15	− 0.06	65			1.04	− 0.14
45	6.52	0.47	1.21	0.00	70			1.05	− 0.13
50			1.29	+ 0.08	75	5.16	0.15	1.05	− 0.13
55			1.32	+ 0.11	80			1.05	− 0.13
60	6.56	0.51	1.37	+ 0.16	85			1.05	− 0.13
65			1.42	+ 0.21	90	5.20	0.19	1.05	0.13
68			1.50	+ 0.29	95			1.05	− 0.13
70			1.51	+ 0.30	100			1.05	− 0.13
75	6.58	0.53	1.57	+ 0.36	105	5.18	0.17	1.05	− 0.13
80			1.65	+ 0.44	110			1.05	− 0.13
85			1.73	+ 0.52	115			1.05	− 0.13
90	6.63	0.58	1.82	+ 0.61	120	5.20	0.19	1.05	− 0.13
95			1.92	+ 0.71	125			1.05	− 0.13
100			1.99	+ 0.78	130			1.05	− 0 13

1	2	3	4	5	1	2	3	4	5
105	6.64	0.59	2.08	−0.87	135	5.19	0.18	1.05	−0.13
110			2.22	−1.01	140			1.05	0.13
115			2.29	−1.08	145			1.05	0.13
120	6.68	0.63	2.43	−1.22	150			1.05	−0.13
125			2.60	−1.39	155			1.05	0.13
130			2.80	−1.59	160	5.20	0.19	1.05	0.13
135	6.69	0.64	3.07	−1.86					
140			3.25	−2.04					
145			3.42	−2.21					
150			3.42	−2.21					
155			3.63	−2.42					
160	6.69	0.64	3.58	−2.37					
180			3.75	−2.54					
225	6.70	0.65	3.85	−2.64					

Digestion experiment No. 8.

Casein solutions: 2.5 g of cow's casein V and human casein 38, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.

Enzyme: mixed gastric juice from infants. 5 ml of the gastric juice was added to 50 ml of casein solution.

Hydrogen ion concentration: solution of cow's casein pH 2.06,
solution of human casein pH 2.12.

Temperature: 37° C.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	5.46	0.00	2	4.04	0.00
13	5.54	0.08	15	4.05	0.01
45	5.58	0.12	45	4.04	0.00
78	5.60	0.14	80	4.07	0.03
149	5.62	0.16	154	4.08	0.04

of 0.1 N sodium hydroxide or 0.3 N hydrochloric acid given in the tables below. A difference in the concentrations of sodium and chlorine ions between the experiments in one series is unavoidable when using this method. Separate control experiments have shown, however, that these differences do not influence the results (digestion experiment No. 15). Commercial pepsin (Parke, Davis & Co 1:3000)as well as crystalline pepsin and gastric juice from infants were used, and the

Digestion experiment No. 9.

Casein solutions: 12.5 g of cow's casein XI[1] and 6.25 g of human casein 40, 62.5 and 31.25 ml of 0.1 N sodium hydroxide, 62.5 and 31.25 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 500 and 250 ml respectively.
Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.
Hydrogen ion concentration: solutions of cow's casein pH 1.42—3.50, solutions of human casein pH 1.76—2.54.
Temperature: 37° C.

pH (initial)	Ml of casein solution	Ml of 0.3 N hydrochloric acid added	Ml of 0.1 N sodium hydroxide added	Ml of H_2O added	Formol titration[2] 0.05 N sodium hydroxide after 2 minutes, ml	D:o after 30 minutes, ml	Difference
			Cow's casein				
1.42	30	4.0	—	6.0	19.28	20.02	0.74
1.60	30	3.0	—	7.0	17.17	17.96	0.79
1.78	30	2.0	—	8.0	—	—	
1.92	30	1.0	—	9.0	13.00	13.96	0.96
2.22	30	—	—	10.0	10.85	11.75	0.90
2.51	30	—	1.0	9.0	9.44	10.06	0.62
2.74	30	—	2.0	8.0	8.86	9.35	0.49
2.78	30	—	3.0	7.0	8.68	9.14	0.46
3.14	30	—	4.0	6.0	7.92	8.36	0.44
3.50	30	—	5.0	5.0	7.14	7.41	0.27
			Human casein				
1.76	25	1.0	—	4.0	14.33	14.66	0.33
1.90	25	0.5	—	4.5	12.76	13.15	0.39
2.05	25	0.3	—	4.7	12.38	12.72	0.34
2.15	25	0.2	—	4.8	11.98	12.34	0.36
2.21	25	—	—	5.0	11.50	11.72	0.22
2.28	25	—	0.2	4.8	10.96	11.19	0.23
2.35	25	—	0.5	4.5	10.82	10.94	0.12
2.54	25	—	1.5	3.5	9.90	10.00	0.10

[1] This preparation was analyzed for phosphorus only, found P, 0.79 %.
[2] 15 ml of digerate used for each titration.

degree of hydrolytic cleavage determined after 30 minutes digestion. The greater part of the digestion process usually takes place within this time. The magnitude of the pH change during this time at optimal hydrogen ion concentration may be seen from experiment No. 14. In some of these experiments (Nos. 9, 10 and 12) the titrations were carried out on 15 ml of digerate.

Digestion experiment No. 10.

Casein solutions: 12.5 g of cow's casein XI and 6.25 g of human casein 40 and 13, 62.5 and 31.25 ml of 0.1 sodium hydroxide, 62.5 and 31.25 ml of 0.3 N hydrochloric acid and distilled water to make the volume 500 and 250 ml respectively.
Enzyme: crystalline pepsin was added in an amount corresponding to 8 mg of enzyme protein per 100 ml of digestion solution.
Hydrogen ion concentration: solutions of cow's casein 1.45 —2.60,
solutions of human casein 1.65—2.78.
Temperature: 37° C.

pH (initial)	Ml of casein solution	Ml of 0.3 N hydrochloric acid added	Ml of 0.1 N sodium hydroxide added	Ml of H_2O added	Formol titration[1] 0.05 N sodium hydroxide after 2 minutes, ml	D:o after 30 minutes, ml	Difference
			Cow's casein				
1.45	30	3	—	2	22.30	23.15	0.85
1.54	30	2	—	3	19.51	20.54	1.03
1.72	30	1	—	4	16.76	18.12	1.36
1.82	30	0.5	—	4.5	15.40	16.61	1.21
1.87	30	0.4	—	4.6	15.53	16.68	1.15
1.98	30	0.3	—	4.7	15.05	16.18	1.13
2.12	30	—	1.0	4.0	13.30	14.18	0.88
2.32	30	—	2.0	3.0	12.16	12.96	0.80
2.60	30	—	3.0	2.0	11.40	12.14	0.74
			Human casein 40				
1.65	lost by accident						
1.83	25	0.5	—	4.5	14.12	14.44	0.32
1.98	25	0.3	—	4.7	13.41	13.80	0.39
2.05	25	—	—	5.0	12.60	12.85	0.25
2.18	25	—	1.0	4.0	11.45	11.70	0.25
2.20	25	—	2.0	3.0	10.58	10.80	0.22
2.78	25	—	3.0	2.0	9.60	9.76	0.16
			Human casein 13				
1.66	30	1.5	—	3.5	16.48	16.94	0.46
1.76	30	1.0	—	4.0	15.28	15.76	0.48
1.85	30	0.5	—	4.5	13.74	14.26	0.52
1.92	30	0.3	—	4.7	13.64	14.18	0.54
1.98	30	0.2	—	4.8	12.68	13.13	0.45
2.02	30	—	—	5.0	12.60	13.02	0.42
2.15	30	—	1.0	4.0	11.62	12.02	0.40
2.31	30	—	2.0	3.0	10.64	11.00	0.36
2.58	30	—	3.0	2.0	9.86	10.18	0.32

[1] 15 ml of digerate used for each titration.

Digestion experiment No. 11.

Casein solutions: 2.5 g of cow's casein XVIII and human casein 13 respectively, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.

Enzyme: mixed gastric juice from infants. 3 ml of gastric juice was added to 18 ml of casein solution.

Hydrogen ion concentration: solution of cow's casein pH 1.48—3.55,
solution of human casein pH 1.45—3.50.

Temperature: 37° C.

pH (initial)	Ml of casein solution	Ml of 0.3 N hydrochloric acid added	Ml of 0.1 N sodium hydroxide added	Ml of H_2O added	Formol titration 0.05 N sodium hydroxide after 2 minutes, ml	D:o after 30 minutes ml	Difference
			Cow's casein				
1.48	15	3.0	—	—	10.18	10.24	0.06
1.61	15	2.0	—	1.0	8.43	8.50	0.07
1.80	15	1.0	—	2.0	6.90	7.27	0.37
2.10	15	—	—	3.0	5.16	5.58	0.42
2.67	15	—	1.5	1.5	4.43	4.70	0.27
3.45	15	—	2.5	0.5	3.90	4.20	0.30
3.55	15	—	3.0	—	3.64	3.92	0.28
			Human casein				
1.45	15	3.0	—	—	9.45	9.50	0.05
1.68	15	1.5	—	1.5	7.15	7.25	0.10
1.79	15	1.0	—	2.0	6.48	6.59	0.11
1.87	15	0.8	—	2.2	6.38	6.50	0.12
1.99	15	0.5	—	2.5	5.52	5.74	0.22
2.10	15	—	—	3.0	4.88	5.06	0.18
2.65	15	—	1.5	1.5	4.06	4.20	0.14
3.50	15	—	3.0	—	3.30	3.42	0.12

Digestion experiment No. 12.

Casein solutions: 12.5 g of cow's casein XVI and 6.26 g of the α-fraction of cow's casein XVI, 62.5 and 31.25 ml of 0.1 N sodium hydroxide, 62.5 and 31.25 ml of 0.3 hydrochloric acid and distilled water to make the volume 500 and 250 ml respectively.

Enzyme: crystalline pepsin was added in an amount corresponding to 8.0 mg of enzyme protein per 100 ml of digestion solution.

Hydrogen ion concentration: solution of cow's casein XVI pH 1.63—2.88. Solution of α-casein pH 1.70—2.94.

Temperature: 37° C.

pH (initial)	Ml of casein solution	Ml of 0.3 N hydrochloric acid added	Ml of 0.1 N sodium hydroxide added	Ml of H_2O added	Formol titration[1] 0.05 N sodium hydroxide after 2 minutes, ml	D:o after 30 minutes, ml	Difference
			Cow's casein XVI				
1.63	25	1.5	—	3.5	19.18	20.38	1.20
1.75	25	1.0	—	4.0	17.32	18.60	1.28
1.86	25	0.7	—	4.3	16.88	18.20	1.32
1.89	25	0.5	—	4.5	15.96	17.33	1.37
1.95	25	0.4	—	4.6	15.58	17.01	1.43
2.00	25	0.3	—	4.7	15.28	16.58	1.30
2.03	25	0.2	—	4.8	14.94	16.22	1.28
2.11	25	—	—	5.0	14.30	15.47	1.17
2.61	25	—	2.0	3.0	12.52	13.40	0.88
2.88	25	—	3.0	2.0	11.96	12.75	0.79
			α-Fraction of cow's casein XVI.				
1.70	25	1.0	—	4.0	17.68	19.18	1.50
1.80	25	0.5	—	4.5	16.71	18.30	1.59
1.88	25	0.3	—	4.7	16.16	17.92	1.76
2.00	25	0.2	—	4.8	15.74	17.54	1.80
2.04	25	—	—	5.0	15.21	16.67	1.46
2.08	25	—	—	5.0	15.38	16.78	1.40
2.15	25	—	0.5	4.5	14.60	16.02	1.42
2.55	25	—	2.0	3.0	13.04	14.50	1.46
2.94	25	—	3.0	2.0	precipitation		

[1] 15 ml of digerate used for each titration.

Digestion experiment No. 13.

Casein solutions: 2.5 g of human casein 38, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.

This solution was divided in three portions of 25 ml each.

I 25 ml of casein solution + 1 ml of water.

II 25 ml of casein solution + a few drops of 1 N sodium hydroxide, and water to 1 ml.

III 25 ml of casein solution + a few drops of 1 N hydrochloric acid and water to 1 ml.

Enzyme: mixed gastric juice from infants. 5 ml of the gastric juice was added to 26 ml of casein solution.

Hydrogen ion concentrations of solutions:

I pH 1.38 II pH 1.93 III pH 3.72.

Temperature: 37° C.

Solution I			Solution II			Solution III		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	9.00	0.00	2	5.82	0.00	2	4.18	0.00
60	9.36	0.36	60	6.22	0.40	60	4.44	0.26
125	9.36	0.36	125	6.33	0.51	120	4.45	0.27
180	9.42	0.42	180	6.44	0.62	180	4.51	0.33
240	9.58	0.58	240	6.50	0.68	240	4.56	0.38

Digestion experiment No. 14.

The variation of the hydrogen ion concentration of solutions of cow's and human casein, prepared in the same manner as in digestion experiment No. 9, and under the same digestion conditions.

Time of digestion in minutes	Cow's casein	Human casein
	pH	pH
Before adding enzyme	1.99	1.90
0.5	2.01	1.90
1	2.08	
2	2.10	1.95
3	2.11	1.98
4	2.13	1.99
7	2.16	2.00
10	2.20	2.00
12	2.20	2.00
14	2.21	2.02
20	2.27	2.07
25	2.29	2.10
30	2.30	2.11

Digestion experiment No. 15.

Casein solutions: 2.5 g of cow's casein V and human casein 13, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.
Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.
Hydrogen ion concentration: solution of cow's casein pH 2.10, solution of human casein pH 2.15.
Temperature: 37° C.

pH	Ml of casein solution	Ml of 0,3 N hydrochloric acid added	Ml of 0.3 N sodium hydroxide added	Ml of H_2O added	Formol titration 0.05 N sodium hydroxide after 2 minutes, ml	D:o after 30 minutes	Difference
				Cow's casein			
2.12	15	2.0	2.0	1.0	4.08	4.30	0.22
2.11	15	1.0	1.0	3.0	4.07	4.30	0.23
2.11	15	0.5	0.5	4.0	4.05	4.29	0.24
2.10	15	0.0	0.0	5.0	4.09	4.32	0.23
				Human casein			
2.15	15	2.0	2.0	1.0	3.64	3.76	0.12
2.16	15	1,0	1.0	3.0	3.62	3.72	0.10
2.14	15	0.5	0.5	4.0	3.65	3.76	0.11
2.15	15	0.0	0.0	5.0	3.62	3.73	0.11

An attempt has also been made to determine the extent to which an acidic casein solution is broken down in the stomach of the infant. It has been found that such *in vivo* experiments are best performed on infants who are recovering from pyloric stenosis. These infants retain a casein solution in the stomach much better than completely healthy children, and there seems to be no reason to suspect any qualitative difference in the peptic activity of the two groups. For two reasons the formol titration method was found unsuitable for following the breakdown of casein in *in vivo* experiments. In the first place, it is not known to what extent breakdown products are resorbed from the stomach, and secondly, the casein solution always becomes diluted with gastric juice. A better way of studying the breakdown of casein solutions *in vivo* is to follow the changes in the electrophoretic pattern of the digestion mixture. From a previous knowledge of the changes in the electrophoretic patterns during *in vitro* digestion with pepsin and gastric juice, some conclusions can be drawn regarding the breakdown *in vivo*. In experiments Nos. 16, 17 and 18 the peptic breakdown of casein solutions *in vitro*, using commercial pepsin and gastric juice, has been

followed electrophoretically, and the results might be compared with those obtained in the *in vivo* experiments Nos. 19, 20, 21 and 22.

For *in vivo* experiments the following procedure was used. From 60 to 150 ml (depending on the infant's ordinary intake) of a 2 % solution of casein adjusted to a pH of about 2.5 were introduced into the stomach by means of a stomach tube. All experiments were performed before the first meal in the morning. To make sure the stomach retained nothing from previous meals, it was emptied by means of the tube before the casein solution was run in. After that, samples of the gastric content, in a volume of about 20 ml each, were taken at regular intervals during the next 30—60 minutes. In order to stop the peptic digestion, the samples were immediately neutralized to pH 6—7 by adding the appropriate amount of 0.3 N sodium hydroxide. The reaction was controlled by means of naphthyl red. At the end of the experiment the hydrogen ion concentration of all samples was checked by means of the glass electrode, and if necessary adjusted to a pH of 7.0—7.2 with 0.1 N sodium hydroxide or hydrochloric acid. During gastric digestion the casein solutions nearly always became opalescent, and a fine precipitate was also often observed. The latter was easily redissolved if, after being neutralized, the samples were shaken for a while. If the samples were contaminated by mucus the latter was removed by filtration through a thin layer of glass wool. The samples were analyzed for total nitrogen. Samples which were not too opalescent were examined electrophoretically. In order to get uniform conditions for electrophoresis, all samples were (before dialysis) diluted to the same protein concentration (usually 0.5 %), as indicated by the figures for total nitrogen. In the interval between the digestion experiments and electrophoresis, samples were kept in a frozen condition at a temperature of — 18° C. It must perhaps be pointed out that, before the electrophoresis experiments, all solutions are dialyzed for 48 hours. It is, therefore, clear that low molecular split-products are removed, and only relatively large parts of the protein molecule remain in the solutions. All electrophoresis experiments were carried out at 0.0° C with a potential gradient of about 5 volts/cm. Phosphate buffers of pH 7.68 and ionic strength 0.15 (phosphate 0.10 + sodium chloride 0.05) were used.

The *in vitro* experiments were carried out in an analogous manner. In digestion experiment No. 16, however, the samples were diluted to a protein concentration of 1.5 % and, not as in the later experiments, to 0.5 %. A buffer of higher ionic strength was also used in this early experiment.

Digestion experiment No. 16.

Casein solutions: 2.5 g of cow's casein V and human casein 13 respectively, 12.5 ml of 0.1 N sodium hydroxide, 12.5 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 100 ml.
Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.
Hydrogen ion concentration: solution of cow's casein pH 2.18,
solution of human casein pH 2.20.
Electrophoresis experiments: 20 ml of the digerate were transferred into a beaker containing 8 ml of 0.1 N sodium hydroxide. This changed the pH to about 7 and the peptic hydrolysis stopped. Phosphate buffers of pH 7.45 and ionic strength 0.25 (phosphate 0.05 and sodium chloride 0.20) were used. The casein solutions were dialysed for 48 hours in the refrigerator against 2 litres of buffer solution before electrophoresis.
Water value of viscosimeter: 11.0 seconds.
Temperature: 30° C.

Cow's casein				Human casein			
Time of enzyme action, minutes	Relative viscosity	Difference from value at zero time	Electrophoretic pattern shown in fig. No.	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Electrophoretic pattern shown in fig. No.
Before adding enzyme	1.64	0.00	18 a	2	4.10	0,00	18 e
18	1.36	−0.28	b	18	4.30	0.20	f
83	5.93	+4.29	c	83	4.35	0.25	g
198	gel formed		d	198	4.39	0.29	h

In the last experiment the gel was homogenized before taking off a sample for electrophoresis. After mixing with the sodium hydroxide the precipitate went into solution.

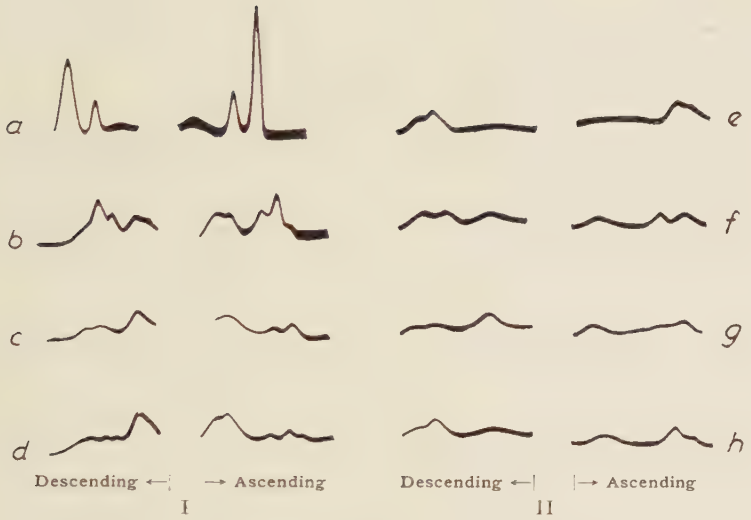

Fig. 18. Electrophoretic patterns obtained at different stages of hydrolysis of cow's (I) and human (II) casein with commercial pepsin. Experimental data are given in digestion experiment No. 16.

Digestion experiment No. 17.

Casein solutions: 5 g of cow's casein XVIII and human casein 20 respectively, 25 ml of 0.1 N sodium hydroxide, 25 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 200 ml.
Enzyme: pepsin (Parke, Davis & Co. 1/3000) 0.05 %.
Hydrogen ion concentration: solution of cow's casein pH 2.5,
solution of human casein pH 2.5.
Electrophoresis experiments: 10 ml of the digerate were transferred into a beaker containing 2 ml of 0.3 N sodium hydroxide and the hydrogen ion concentration adjusted to pH 7.0—7.2 by means of a few drops of 0.1 N sodium hydroxide or hydrochloric acid. The solutions obtained in this way were dialyzed for two days against two litres of phosphate buffer of pH 7.68 and ionic strength 0.15 (phosphate 0.10 + sodium chloride 0.05). Before the electrophoresis the samples were diluted to a protein concentration of 0.5 %.
Temperature: 37° C.

Cow's casein				Human casein			
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Electrophoretic pattern shown in fig. No.	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Electrophoretic pattern shown in fig. No.
Before adding enzyme	5.12		19 a	Before adding enzyme	4.72		19 g
2	5.10	0.00		2	4.72	0.00	
5	5.16	0.06	b	5	4.74	0.02	h
10	5.20	0.10	c	10	4.78	0.06	i
20	5.29	0.19	d	20	4.80	0.08	j
30	5.35	0.25	e	30	4.82	0.10	k
45	5.38	0.28	f	45	4.87	0.15	l

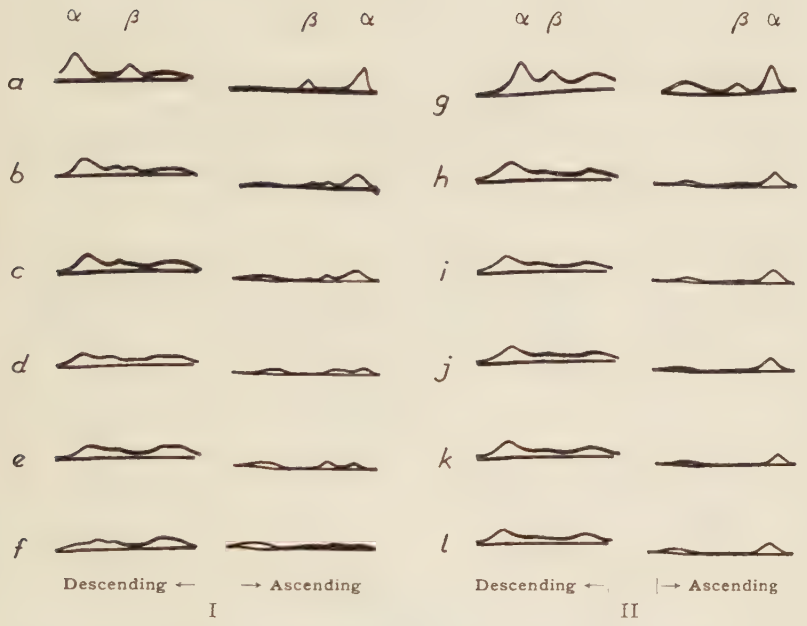

Fig. 19. Electrophoretic patterns obtained at different stages of hydrolysis of cow's (I) and human (II) casein with commercial pepsin. Experimental data are given in digestion experiment No. 17.

Digestion experiment No. 18.

Casein solutions: 2.5 g of cow's casein XI and human casein 11[1] respectively, 10 ml of 0.1 N sodium hydroxide, 10 ml of 0.3 N hydrochloric acid and distilled water to make the volume 100 ml.

Enzyme: mixed gastric juice from infants. 9 ml of the gastric juice was added to 45 ml of the casein solution.

Hydrogen ion concentration: solution of cow's casein pH 2.20,
solution of human casein pH 2.20.

Electrophoresis experiments: 5 ml of the digerate was transferred into a beaker containing 20 ml of phosphate buffer of pH 7.68 and ionic strength 0.15 (phosphate 0.10 + sodium chloride 0.05). The solutions obtained in this way were dialysed for two days against 2 litres of the same buffer.

Temperature: 37° C.

Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at 2 minutes	Electrophoretic pattern shown in fig. No.
	Cow's casein		
Before adding enzyme			20 a
2	5.64	0.00	—
7			b
15	5.82	0.18	c
30	6.04	0.40	d
60	6.15	0.51	—
	Human casein		
Before adding enzyme			20 e
2	4.82	0.00	f
15	5.02	0.20	—
30	5.08	0.26	—
60	5.10	0.28	g

[1] This preparation was analyzed for phosphorus only, found P, 0.40 %.

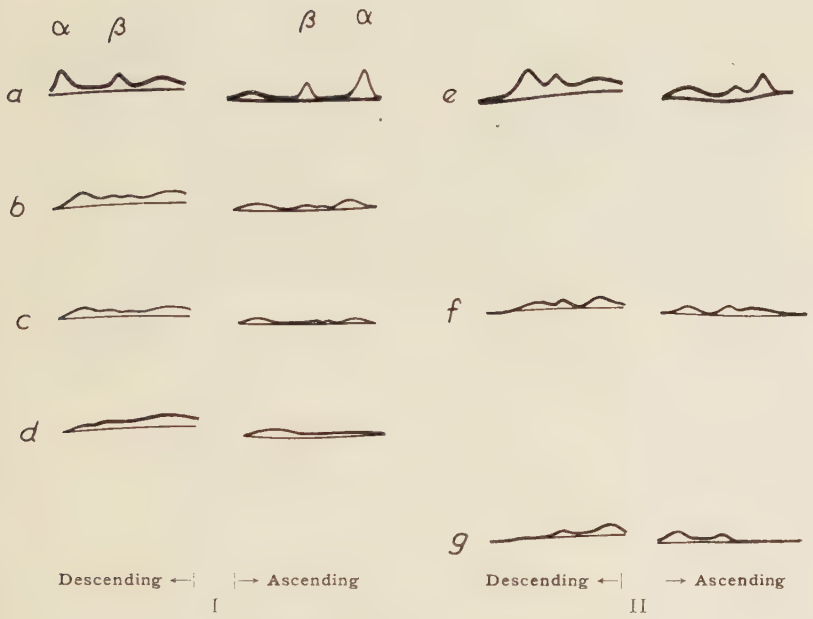

Fig. 20. Electrophoretic patterns obtained at different stages of hydrolysis of cow's (I) and human (II) casein with gastric juice from infants. Experimental data are given in digestion experiment No. 18.

Digestion experiment No. 19.

Casein solutions: 5 g of cow's casein XI and human casein 47 respectively, 20 ml of 0.1 N sodium hydroxide and 20 ml of 0.3 N hydrochloric acid. A further portion of 5 ml of 0.1 N sodium hydroxide was added to both solutions before filling up with water to 200 ml.

Hydrogen ion concentration: solution of cow's casein pH 2.5,
solution of human casein pH 2.7.

140 ml of the above solutions were introduced by means of a stomach tube in the mornings of two consecutive days into the empty stomach of an infant aged 4 months which had recently suffered from pyloric stenosis.

The samples were neutralized and analyzed as described on page 159.

Sample No.	Digestion time, minutes	Volume of sample, ml	Electrophoretic pattern shown in Fig. No.
	Cow's casein		
0	0	10	21 a
1	5	10	—
2	10	10	b
3	15	10	c
4	30	10	d
5	60	10	—
	Human casein		
0	0	10	21 e
1	5	10	—
2	10	10	f
3	15	10	g
4	30	10	—
5	60	10	h

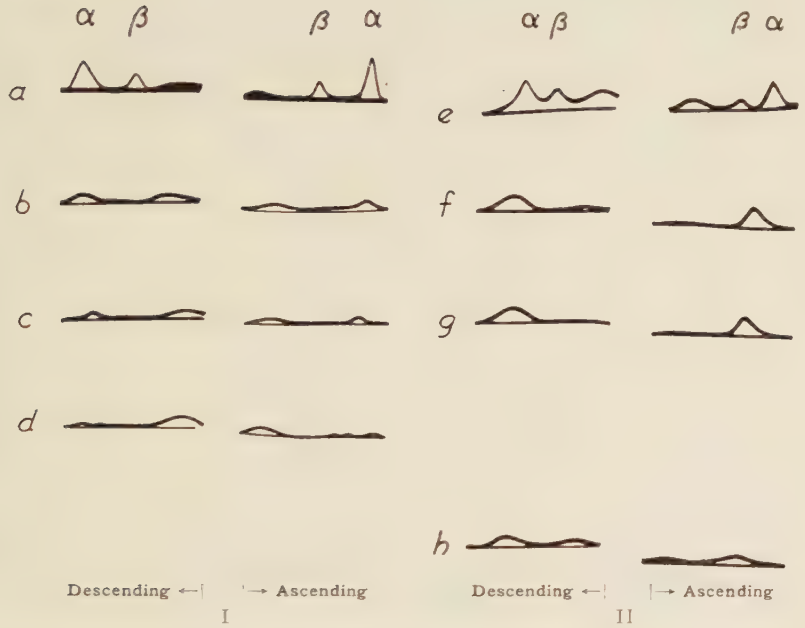

Fig. 21. Electrophoretic patterns obtained at different stages of *in vivo* digestion of cow's (I) and human (II) casein. Experimental data are given in digestion experiment No. 19.

Digestion experiment No. 20.

Casein solutions: 5 g of cow's casein XVIII and human casein 13 respectively, 25 ml of 0.1 N sodium hydroxide and 25 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 200 ml.

Hydrogen ion concentration: solution of cow's casein pH 2.5,
solution of human casein pH 2.5.

100 ml of the above solutions were introduced by means of the stomach tube in the mornings on two consecutive days into the empty stomach of an infant aged 13 months, suffering from hydrocephalus.

The samples were neutralized and analyzed as described on page 159.

Sample No.	Digestion time, minutes	Volume of sample, ml	Electrophoretic pattern shown in Fig. No.
		Cow's casein	
0	0	20	22 a
1	10	20	b
2	20	20	c
3	30	20	d
4	45	6	—
		Human casein	
0	0	20	22 e
1	10	20	f
2	20	20	g
3	30	20	h
4	45	10	—

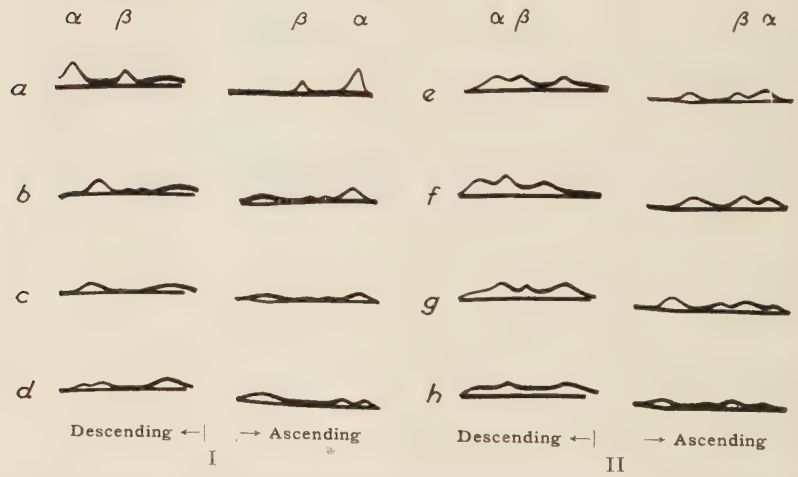

Fig. 22. Electrophoretic patterns obtanined at different stages of *in vivo* digestion of cow's (I) and human (II) casein. Experimental data are given in digestion experiment No. 20.

Digestion experiment No. 21.

Casein solution: a solution of human casein 13 was prepared in the same manner as described in experiment No. 20.

Hydrogen ion concentration: pH 2.5.

80 ml of the above solution were introduced by means of the stomach tube in the morning into the empty stomach of an infant aged 3 months who was a convalescent from pyloric stenosis.

The samples were neutralized and analyzed as described on page 159.

Sample No.	Digestion, time, minutes	Volume of sample, ml	Electrophoretic pattern shown in Fig. No.
0	0	20	23 a
1	10	20	b
2	20	20	c

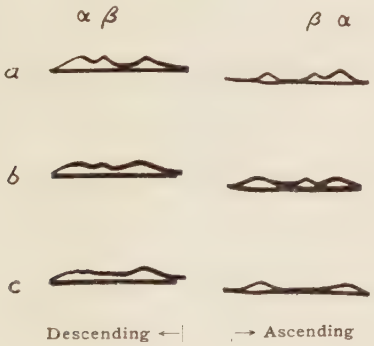

Fig. 23. Electrophoretic patterns obtained at different stages of *in vivo* digestion of human casein. Experimental data are given in digestion experiment No. 21.

Digestion experiment No. 22.

Casein solution: a solution of cow's casein XVIII was prepared in the same manner as described in experiment No. 20.

Hydrogen ion concentration: pH 2.5.

100 ml of the above solution were introduced in the usual manner into the stomach of an infant aged 13 months, suffering from hydrocephalus.

The samples were neutralized and analyzed in the usual manner.

Sample No.	Digestion, time minutes	Volume of sample, ml	Electrophoretic pattern shown in Fig. No.
0	0	20	24 a
1	10	20	b
2	20	20	c
3	30	20	d
4	45	8	—

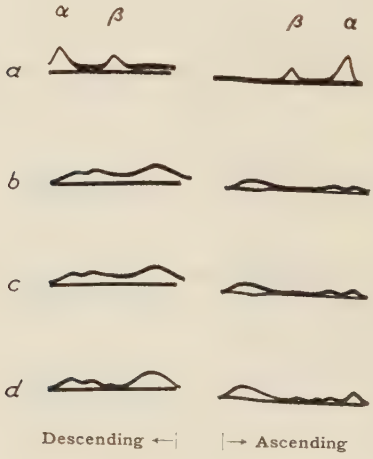

Fig. 24. Electrophoretic patterns obtained at different stages of *in vivo* digestion of cow's casein. Experimental data are given in digestion experiment No. 22.

The tryptic digestion of cow's and human casein

A number of comparative experiments, analogous to the pepsin experiments described above, have been carried out on the tryptic digestion of the two caseins.

For reasons which will be discussed later the author has also carried out comparative experiments on the tryptic digestion of casein in the presence of phosphatase added at different stages of digestion.

The trypsin used was a commercial preparation by Difco (1/110). No crystalline trypsin was available. The Difco preparation was used in amounts of 0.1 g per 100 ml of digerate.

Table 10. *Proteolytic activity of the different phosphatase preparations.*

	Cow's casein			Human casein		
	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
Calf's bone phosphatase	2	1.39	0.00	2	1.05	0.00
	30	1.42	0.03	30	1.08	0.03
	60	1.44	0.05	60	1.08	0.03
Calf's intestinal phosphatase	2	3.60	0.00	2	3.71	0.00
	30	3.65	0.05	30	3.74	0.03
	60	3.70	0.10	60	3.75	0.04
Human intestinal phosphatase	2	1.54	0.00	2	1.15	0.00
	30	1.56	0.02	30	1.17	0.02
	60	1.56	0.02	60	1.18	0.03

The bone phosphatase preparations used were prepared according to Martland and Robison (1929). Intestinal phosphatase was also prepared from the intestines of both calves and infants, according to Schmidt and Thannhauser (1943). On account of the larger amount of intestinal mucosa available the phosphatase preparation from calf's intestines could be purified to a higher degree than that from infants. The activity of the different preparations was determined by the method

Digestion experiment No. 23 (cf. Fig. 25).

Casein solutions: 5 g of cow's casein X and human casein 39 respectively, 25 ml of 0.1 N sodium hydroxide and distilled water to bring the volume up to 200 ml.
Hydrogen ion concentration: solution of cow's casein pH 7.50,
solution of human casein pH 7.51.
Enzyme: trypsin (Difco) 0.1 %.
Temperature: 37° C.
Water values of viscosimeters: 11.2 and 20.4 seconds respectively.

Cow's casein				Human casein			
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at zero time	Relative viscosity	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at zero time	Relative viscosity
Before adding enzyme	1.18	0.00		Before adding enzyme	1.22	0.00	
3			1.16	4			1.18
5			1.16	6			1.16
9	1.88	0.70		11	1.92	0.70	
14			1.07	15			1.13
16	2.30	1.12		20	2.16	0.94	
22			1.07	23			1.13
28			1.12	29			1.13
				31			1.10
30			1.16 Fine precipitate	37	2.42	1.20	
				40			1.11
				48	2.56	1.34	
32			1.16	52			1.11
34	3.04	1.86		61	2.70	1.48	
39			1.16	70			1.10
46	3.30	2.12		83	2.78	1.56	
51			1.07	93			1.08
58	3.52	2.34		118	3.06	1.84	
60			1.07	120			1.08
71			1.07				No precipitate was observed during the digestion
80	3.86	2.68					
94			1.07	170	3.34	2.12	
110	4.24	3.06	Precipitate redissolved				
128			1.07	175			1.08
165	4.86	3.68					
174			1.07				

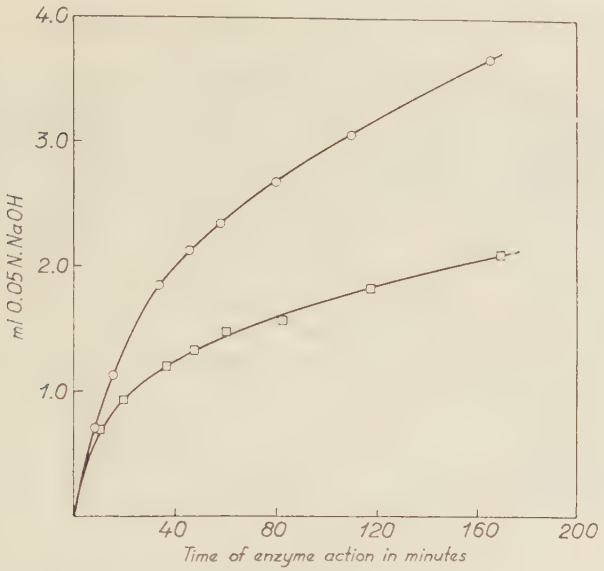

Fig. 25. Hydrolysis of cow's (O) and human (□) casein with commercial trypsin. Experimental data are given in digestion experiment No. 23.

of King and Armstrong (1934) as modified by Binkley, Shank and Hoagland (1944). A unit of alkaline phosphatase is that amount of activity which, under standard conditions given by the latter authors, will within 30 minutes liberate 1 mg of phenol from the phenyl phosphate used as substrate. The activities of the different preparations are shown below.

Source of phosphatase	Units of phosphatase per 100 ml
Calf's bones	80
Calf's intestines	280
Intestines from infants	220

The phosphatase preparations were tested for proteolytic activity. To 50 ml of human and cow's casein solution (about 2 % of pH 8.0 prepared in the usual manner) was added 0.5 ml of the phosphatase solution. As indicated in Table 10 only very weak proteolysis occurred.

Digestion experiment No. 24.

Casein solutions: 10 g of cow's casein XVII and human casein 57 (water content of both caseins unknown) respectively, 50 ml of 0.1 N sodium hydroxide, 50 ml of 0.3 N hydrochloric acid and distilled water to bring the volume up to 500 ml. The protein concentration of these solutions was calculated from nitrogen determinations (Kjeldahl's method), and was 1.8 % in each. 100 ml of the solution of cow's casein were added to 13 ml of 0.3 N sodium hydroxide and 100 ml of the human casein solution to 11 ml of 0.3 N sodium hydroxide and 2 ml water. The protein concentration of these latter solutions was therefore about 1.6 %.
Enzyme: trypsin (Difco) 0.1 %.
Hydrogen ion concentration: solution of cow's casein pH 8.40,
solution of human casein pH 8.40.
Temperature: 37° C.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	1.18	0.00	2	0.78	0.00
15	1.47	0.29	15	1.22	0.44
30	1.98	0.80	30	1.49	0.71
60	2.44	1.26	60	1.72	0.94
90	2.74	1.56	90	1.90	1.12
120	3.02	1.84	120	2.10	1.32
150	3.31	2.13	150	2.21	1.43
180	3.46	2.28	180	2.32	1.54

Discussion and Summary of Chapter III

During digestion of human casein with pepsin or gastric juice the author has observed no formation of pseudonuclein under the experimental conditions used. This agrees with the findings of Wróblewski and others. The only visible change in the solutions of human casein when digested with pepsin or gastric juice is an increase in the opalescence of the solutions. The increase in viscosity during peptic digestion of cow's casein has no counterpart in the digestion of human casein (cf. digestion experiments Nos. 1, 2, 5, 6 and 7). If the author's results for the formol titration and the viscosity measurements respectively are plotted on a graph, in a manner similar to that used for cow's casein by Holter, Linderström-Lang and Funder (1932), the difference between the two caseins becomes quite apparent (cf. Figs. 15 and 17). The curves obtained by the author for cow's casein are practically identical with those of Holter, Linderström-Lang and Funder. The

course of the viscosity changes is also the same when gastric juice is employed.

Cow's casein and human casein prepared from cow's and human milk respectively by essentially similar methods are markedly different in their behaviour vis à-vis proteolytic enzymes and gastric juice from infants. It is somewhat surprising to find that human casein is more difficult to digest. The digestion experiments Nos. 1—11 demonstrate this difference in the digestibility of the two caseins. *Both with pepsin (commercial and crystalline) and gastric juice from infants there is obtained a markedly higher degree of cleavage in the case of cow's casein.* The maximum values obtained in digestion experiments Nos. 1—8 are 2—3 times greater for cow's casein than for human casein, both when commercial pepsin and when gastric juice are employed. It is evident from experiment No. 10 that a difference of the same order of magnitude is also obtained when crystalline pepsin is used. This difference is also fairly independent of the hydrogen ion concentration in all experiments except No. 9, where the difference increases towards the alkaline side (in this particular experiment the formol titration values for human casein at the most alkaline pH:s are small, however). The reason for the difference may be that a larger part of the human casein is converted during digestion into phosphorylated peptides resistant to proteolytic enzymes.

The digestion experiments Nos. 9—13 show that the optimal hydrogen ion concentration for the peptic breakdown of the two caseins cannot be appreciably different.

From digestion experiment No. 12 may be seen that α-casein from cow's casein in the pH-range examined (1.7 2.9) is more easily digested than the total casein. HOLTER, LINDERSTRÖM-LANG and FUNDER, when digesting casein fractions of different phosphorus content also found that the fractions containing most phosphorus were more easily digested with pepsin than fractions of lower phosphorus content. There thus seems to be a correlation between phosphorus content and digestibility for native casein fractions.

The electrophoretic patterns of both caseins in different stages of digestion have been studied. Figs. 18 and 19 (digestion experiments Nos. 16 and 17) show that a remarkably rapid change occurs during the initial stages of the peptic hydrolysis. For cow's casein, the most striking changes are the decrease of the peaks due to the α- and β-fraction, and the formation of a new peak with very low mobility. For human casein, on the other hand, at least one of the original peaks remains, even after digestion for 198 and 45 minutes respectively. Owing to the small difference in mobility between the α- and β-peaks of human casein it cannot be decided whether the remaining peak corresponds to the original α- or β-fraction of the casein. When human casein is digested, a new peak of low mobility is also formed (more pronounced

Digestion experiment No. 25 (cf. Fig. 26).

Casein solutions: 125 ml portions of the casein solutions prepared in digestion experiment No. 2 were made alkaline by pouring into sodium hydroxide (about 12 ml of 0.1 N) and distilled water added to bring the volume up to 140 ml. As the protein concentration of the original solutions was 1.7 % the protein concentration after dilution was about 1.5 %.
Proteolytic enzyme: trypsin (Difco) 0.1 %.
Phosphatase: preparation from calf's intestines.
Hydrogen ion concentration: solution of cow's casein 8.50,
solution of human casein 8.50.
Temperature: 37° C.

When digestion had proceeded for 120 minutes the phosphatase preparation was added and immediately (within 2 minutes) a new formol titration was made. The digestion occurring between the last titration before, and the first one after adding the phosphatase, was assumed to be negligible.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	0.89	0.00	2	0.92	0.00
30	1.75	0.86	30	1.43	0.51
60	2.04	1.15	60	1.80	0.88
90	2.33	1.44	90	1.93	1.01
120	2.54	1.65	120	2.14	1.22
To 50 ml of the digerate was added ½ ml of phosphatase preparation from calf's intestines			To 50 ml of the digerate was added ½ ml of phosphatase preparation from calf's intestines		
121	4.70	1.65	121	4.17	1.22
136	5.12	2.07	136	4.56	1.61
151	5.25	2.20	151	4.77	1.82
211	5.59	2.54	211	5.21	2.26
241	5.74	2.69	241	5.40	2.45

after prolonged digestion), cf. Fig. 6 in the author's preliminary report (1945). When gastric juice from infants was used as source of enzyme, similar changes in the electrophoretic patterns were observed (cf. Fig. 20, digestion experiment No. 18). That casein solutions may undergo similar changes in the electrophoretic structure when digested *in vivo* in the stomach of an infant as when digested *in vitro* is seen from experiment No. 19 (Fig. 21). This experiment was performed on a child which usually had very active gastric juice. The electrophoretic structure of the casein is in this experiment changed in the same manner as when digested with pepsin. It is evident also from digestion experiment No. 20, that the casein structure is altered during digestion in the

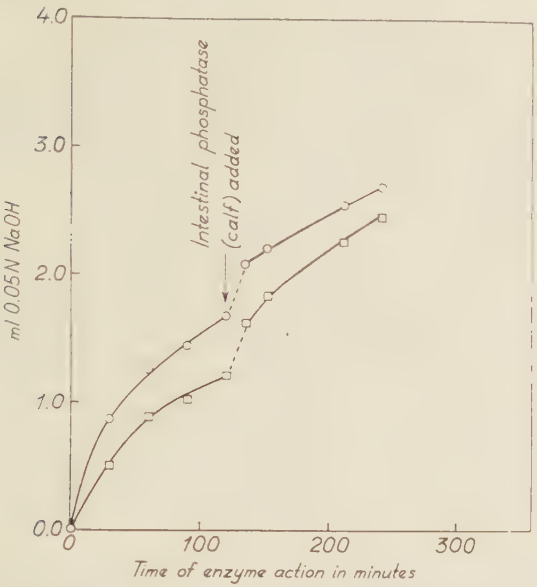

Fig. 26. Hydrolysis of cow's (○) and human (□) casein with commercial trypsin and phosphatase from calf's intestines. Experimental data are given in digestion experiment No. 25.

stomach, though the differences between human and cow's casein are not so pronounced as in experiment No. 19. This may be due to the comparatively low proteolytic activity of the gastric juice of the infant used in experiment 20. This infant was suffering from hydrocephalus. The same infant was also used for experiment No. 22, performed only with cow's casein solution. It is seen that small peaks still remain after 45 minutes digestion in the stomach. Experiment No. 21 on the other hand, was performed with human casein on an infant whose gastric juice was usually very active, and shows a result similar to the human casein part of experiment No. 19. The electrophoretic experiments performed thus indicate that a breakdown of the casein molecule may actually occur in the stomach of the infant under the experimental conditions used.

Just as in digestion with pepsin, there is a marked difference between human and cow's casein when digested with trypsin, human casein being more resistant vis-à-vis the enzymes present in commercial trypsin (Fig. 25, digestion experiments Nos. 23 and 24). No differences between the two caseins with regard to the viscosity changes were observed during the tryptic digestion.

Digestion experiment No. 26 (cf. Fig. 27).

Casein solutions: 80 ml portions of the acid casein solutions prepared in experiment No. 24 were made alkaline by pouring into sodium hydroxide (9—10 ml) and distilled water added to bring the volume up to 90 ml. As the protein concentration of the original solutions was 1.8 % the protein concentration after dilution with sodium hydroxide was about 1.6 %.

Proteolytic enzyme: trypsin (Difco) 0.1 %.

Phosphatase: preparation from calf's intestines.

Hydrogen ion concentration: solution of cow's casein pH 8.50,
solution of human casein pH 8.50.

Digestion procedure: to 90 ml of the above casein solutions was added trypsin (0.09 g). After digestion for 5 hours which was followed by means of formol titrations (for these c:a 40 ml of the digerates had to be taken) 50 ml of the digestion mixtures remained. To this volume were added 0.5 ml of neutralized phosphatase solution, 1 ml of 1 % magnesium chloride and 1.5 ml of 0.1 N sodium hydroxide, to bring the pH (which was now about 7) back to 8.5. After further digestion for about 4 hours, another portion of 0.5 ml of phosphatase solution and 1.5 ml of 0.1 N sodium hydroxide were added to the digerates. Both additions of phosphatase etc. were made immediately after taking a sample for formol titration and they were also immediately followed by new formol titrations. It was assumed that in the short time between these two formol titrations no appreciable proteolysis occurred.

Temperature: 37° C.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	0.86	0.00	2	1.00	0.00
15	1.51	0.65	15	1.48	0.48
30	1.94	1.08	30	1.81	0.81
90	2.70	1.84	90	2.25	1.25
150	3.22	2.36	150	2.54	1.54
210	3.48	2.62	210	2.78	1.78
270	3.75	2.89	270	2.88	1.88
300	3.92	3.06	300	3.00	2.00
Immediately after this titration phosphatase etc. were added			Immediately after this titration phosphatase etc. were added		
305	5.04	3.06	305	4.54	2.00
335	5.36	3.38	335	4.76	2.22
350	5.41	3.43	350	4.88	2.34
410	5.67	3.69	410	5.14	2.60
470	5.72	3.74	470	5.58	3.04
530	5.92	3.94	530	5.72	3.18
Immediately after this titration phosphatase etc. were added			Immediately after this titration phosphatase etc. were added		
535	8.24	3.94	535	8.10	3.18
620	8.60	4.30	600	8.84	3.92
680	8.66	4.36	660	9.22	4.30

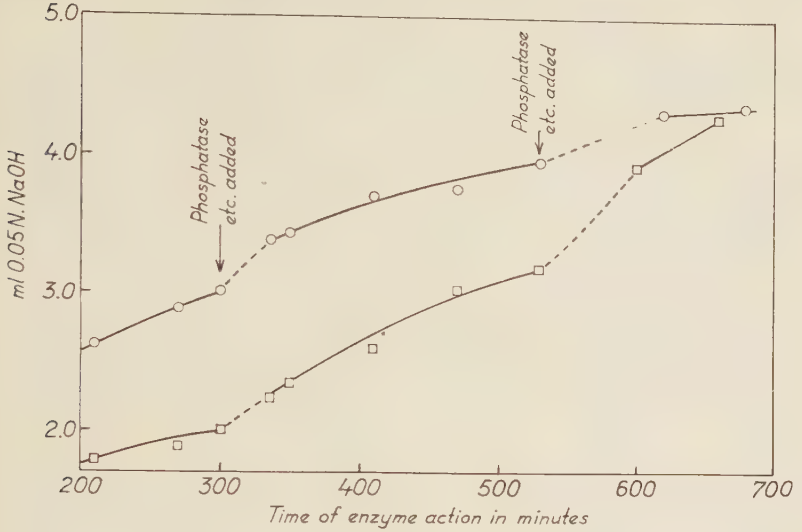

Fig. 27. Hydrolysis of cow's (O) and human (□) casein with commercial trypsin. Phosphatase from calf's intestines added at times indicated. Experimental data are given in digestion experiment No. 26.

As pointed out above, a possible explanation for the differences in digestibility found, would be that the two caseins have different tendencies to form "enzyme-resistant" peptides of phosphopeptone type during digestion. If this assumption is correct, it might be expected that an addition of phosphatase to the casein solutions during tryptic digestion would tend to remove the difference found. As is seen from digestion experiments Nos. 25 and 26 (Figs. 26 and 27), the diagrams for the tryptic cleavage after adding phosphatase from calf's intestines become convergent. Digestion experiment No. 27, (Fig. 28) shows that when inactivated phosphatase is added, the diagrams remain parallel As is seen from the tables the addition of phosphatase solution immediately causes an appreciable increase in the amount of 0.05 N sodium hydroxide required for the formol titration. This is because the solutions of phosphatase are somewhat acid and also contain substances which exert a buffer action. The increase represented by the dotted lines in the figures cannot be attributed solely to the phosphatase action, since a similar though less pronounced increase is also found in the control experiment (Fig. 28, first addition of phosphatase). A possible cause of this increase is that the trypsin already present in the solution becomes activated by a factor present in heated phosphatase solution also.

Digestion experiment No. 27 (cf. Fig. 28).

All experimental conditions as in the preceding experiment but heat inactivated instead of active phosphatase solution was used.

Cow's casein			Human casein		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	1.24	0.00	2	0.87	0.00
15	1.81	0.57	15	1.22	0.35
60	2.76	1.52	30	1.44	0.57
90	3.06	1.82	90	2.12	1.25
150	3.55	2.31	150	2.42	1.55
210	3.78	2.54	210	2.72	1.85
270	4.06	2.82	270	2.84	1.97
300	4.08	2.84	300	2.89	2.02
Immediately after this titration inactivated phosphatase etc. were added			Immediately after this titration inactivated phosphatase etc. were added		
305	5.19	2.84	305	5.21	2.02
390	5.56	3.21	390	5.72	2.53
510	5.66	3.31	510	5.90	2.71
Immediately after this titration inactivated phosphatase etc. were added			Immediately after this titration inactivated phosphatase etc. were added		
515	6.60	3.31	515	7.72	2.71
570	6.90	3.61	570	7.74	2.73

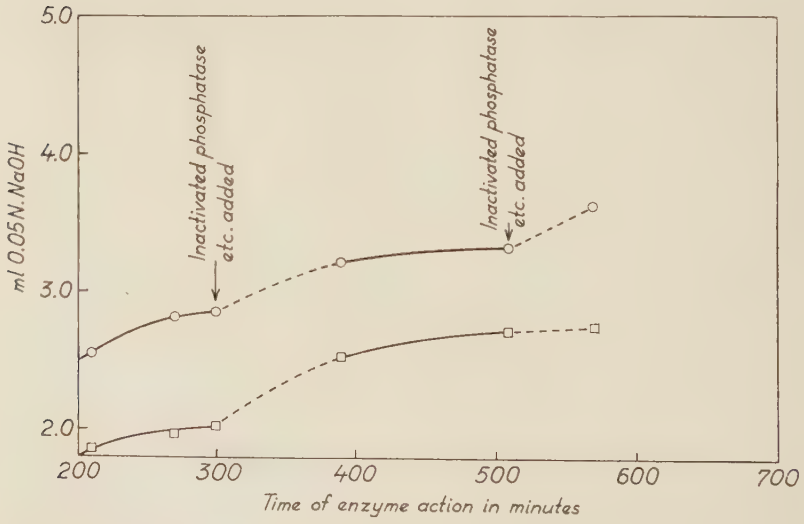

Fig. 28. Hydrolysis of cow's (○) and human (□) casein with commercial trypsin. Heat inactivated phosphatase from calf's intestines added at times indicated. Experimental data are given in digestion experiment No. 27.

Digestion experiment No. 28 (cf. Fig. 29).

Casein solution: the same solution of cow's casein as used in digestion experiment No. 24 (protein concentration 1.6 %).
Proteolytic enzyme: trypsin (Difco) 0.1 %.
Phosphatase: preparation from calf's bones.
Hydrogen ion concentration: pH 8.40. *Temperature:* 37° C.

Digestion mixture A: to 70 ml of the casein solution were added trypsin (0.07 g) and 0.7 ml of active phosphatase solution. Digestion mixture B: to 70 ml of the casein solution were added trypsin as in A and 0.7 ml of heat-inactivated phosphatase solution.

A			B		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
2	2.88	0.00	2	2.76	0.00
15	3.39	0.51	15	3.24	0.48
30	3.87	0.99	30	3.70	0.94
60	4.37	1.49	60	4.16	1.40
90	4.88	2.00	90	4.52	1.76
120	5.22	2.34	120	4.81	2.05
150	5.51	2.63	150	5.01	2.25
180	5.76	2.88	180	5.23	2.47
270	6.44	3.56	270	5.66	2.90
510	7.34	4.46	510	6.22	3.46

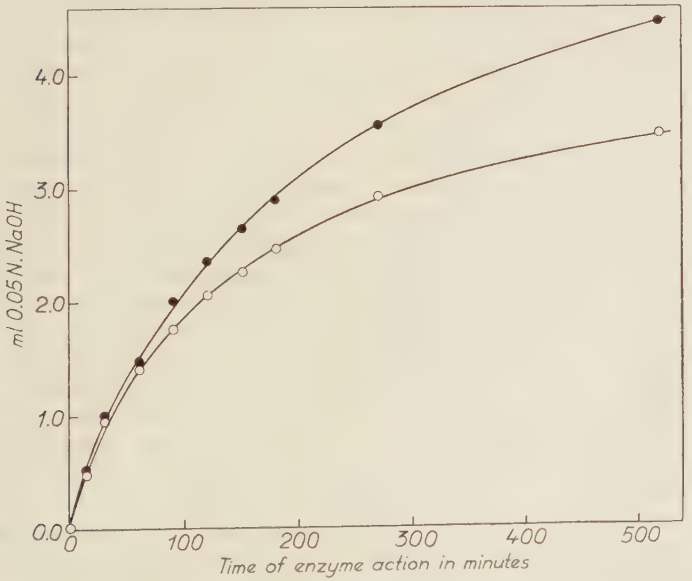

Fig. 29. Hydrolysis of cow's casein with commercial trypsin in the presence of active (●) and heat inactivated (○) phosphatase from calf's bones. Experimental data are given in digestion experiment No. 28.

Experiment No. 28 (Fig. 29) shows that when phosphatase is added to the tryptic digestions at the beginning of the proteolysis, it has no effect on the degree of hydrolysis in the first part of digestion. After 1 2 hours, however, the presence of phosphatase causes a marked increase of the titration values. This result tallies well with some findings of other authors. For example, RIMINGTON and KAY (1926) have shown that phosphatase causes no dephosphorylation of intact casein. Only the proteolytic breakdown products are sensitive towards phosphatase (cf. also LÖVGREN 1945).

CHAPTER IV

Enzyme-resistant phosphopeptones from cow's and human casein

Review of literature. S. POSTERNAK (1923) appears to have been the first to isolate an organic compound containing phosphorus from the tryptic digests of cow's casein. The first isolated compound contained 5.86 % P and 11.9 % N. POSTERNAK (1927) calls substances of this type lactotyrines, and found that their N/P ratio varied between 3.75 and 4.50. They formed water soluble alkali and alkaline earth salts. After acid hydrolysis he reported the isolation of glutamic acid, aspartic acid, serine and isoleucine. As the only hydroxy-amino-acid was serine he considered it likely that phosphoric acid is bound to the hydroxyl group of this amino acid. This has later been confirmed by LIPMAN (1933) who prepared serine phosphoric acid directly from casein, and by LEVENE and HILL (1933) who isolated phosphoseryl glutamic acid from phosphopeptone prepared according to POSTERNAK. Without being aware of POSTERNAK's work RIMINGTON and KAY (1926) described a compound isolated from tryptic digests of cow's casein, which they gave the name of phosphopeptone. This name more adequately expresses the nature of the compound than POSTERNAK's name of lactotyrine and has therefore come into common use. The phosphopeptone has been the subject of later investigations by RIMINGTON (1927 a, b, 1941), who was the first to give a detailed description of a suitable procedure for the preparation. The substance was prepared in the main as follows. Cow's casein was digested with trypsin at a pH of about 8.5 for 38 hours at a temperature of 37°. The digested mixture was then acidified with acetic acid to a pH of 4.5. The precipitate was removed by filtration, and phosphopeptone removed from the filtrate by means of precipitation with lead acetate. The precipitate was washed, suspended in water and decomposed by hydrogen sulphide. Lead sulphide was removed by filtration, and ex-

cess hydrogen sulphide by means of aeration. Inorganic phosphorus was removed from the solution with magnesia mixture, and the phosphopeptone precipitated as uranium salt. The uranium salt was transformed into copper salt. The yield of copper salt from one kg of casein was 55.5 g. The compound was purified by repeated precipitation, until the N/P ratio did not change on further treatment. The amount of phosphopeptone so obtained represented about 50 % of the total organic phosphorus in the original digerate. It had the following composition: C 28.63, H 4.18, O 34.68, N 7.84, P 6.05, Cu 18.62 %. The N P ratio was 3. From this substance a free phosphopeptone was prepared having the following composition: C 37.44, H 5.75, N 10.13, P 7.05 %, amino nitrogen 1/9 of total nitrogen. The barium salt was also prepared. It had the following composition: N 6.25, P 4.60 and Ba 32.26 %. RIMINGTON reports that the following amino-acids were isolated from the phosphopeptone: "hydroxyglutamic acid", hydroxyaminobutyric acid and serine.

In view of the rather different properties exhibited by the compounds obtained by RIMINGTON and S. POSTERNAK, RIMINGTON and KAY considered (cf. Rimington 1927 b, note on p. 1193) that the compounds were quite different. POSTERNAK (1928) prepared phosphopeptones according to RIMINGTON's method, and found a N/P ratio of 3.5. On hydrolysis he obtained the same amino-acids as found in his own lactotyrines. In 1934 SCHMIDT isolated, after four week's digestion of casein with pancreatic extract, a barium salt of phosphopeptone. Most of the preparations had a N/P ratio of 2. On hydrolysis, the amino-acids serine and glutamic acid were obtained. Prior to 1941 the preparation of phosphopeptone had always been carried out by means of direct tryptic digestion of casein. In that year, however, DAMODARAN and RAMACHANDRAN (1941) prepared enzyme-resistant phosphopeptone by tryptic digestion of pseudonuclein obtained by peptic digestion of casein. They obtained, from different preparations, barium salt of phosphopeptones with a fairly constant composition. They isolated glutamic acid, isoleucine, and serine from the acid hydrolysis of the phosphopeptone. Other hydroxy or dicarboxy-amino-acids were indirectly demonstrated to be absent, and the phosphopeptone, according to DAMODARAN and RAMACHANDRAN, contains 3 molecules of glutamic acid, 3 of isoleucine and 4 of serine. In the same year both T. POSTERNAK and POLLACZEK (1941) and RIMINGTON (1941) published new investigations dealing with the composition of phosphopeptones. RIMINGTON has now come to a different conclusion than previously, and considers the peptide to contain glutamic acid, isoleucine and serine. T. POSTERNAK and POLLACZEK describe the preparation of two compounds called phosphopeptone I and phosphopeptone II respectively. The former was the result of two to five hours' tryptic digestion of casein and phosphopeptone II was obtained after very prolonged tryptic

Table 11. *Composition of phosph*

Author	Year	Preparad as		C %	H %	N %	P %
S. Posternak	1927	free phosphopeptone				11.03	6.51
						11.36	6.29
						11.9	5.86
Rimington	1927	free phosphopeptone		37.44	5.75	10.13	7.05
S. Posternak[1]	1928	free phosphopeptone					
Levene and Hill	1933	sodium salt					
Schmidt	1934	barium salt					
Rimington	1941	free phosphopeptone		35.29	6.16	9.86	7.27
				37.58	6.16	10.40	6.88
T. Posternak and Pollaczek	1941	barium salt	I			6.68	4.16
			II			7.36	6.03
						6.04	5.66
Lowndes, Macara and Plimmer	1941	sodium salt				10.56	5.77
Damodaran and Ramachandran	1941	barium salt				6.48	4.53
						6.44	4.26
						6.39	4.58
						6.46	4.34

[1] The phosphopeptone prepared according to Rimington.

digestion in the manner described by SCHMIDT. Their composition may be seen from Table 11. The phosphopeptones were resistant to aminopeptidases from swine's intestines. The action of kidney phosphatase from swine led to a complete dephosphorylation of phosphopeptone II, but in the case of phosphopeptone I only two thirds of the phosphoric acid residues were removed. The authors point out that the resistance towards aminopeptidase depends on the phosphorylation, and show that a rapid proteolysis sets in at a certain stage during the dephosphorylation by means of kidney phosphatase. This agrees with results published by DAMODARAN and RAMACHANDRAN (1941) who showed that the resistance of the phosphopeptones to trypsin decreases after dephosphorylation by means of alkali. The resistance of the phosphopeptones is thus intimately connected with the organically bound phosphoric acid. When comparing these findings with the higher

eptones prepared by different authors.

Ba %	N/P atomic ratio	Amino nitrogen %	Number of amino acid residues	Amino acid composition suggested
	3.75—4.50		15?	3 glutamic acid, (0.6) aspartic acid, 3 serine, 3 *iso*leucine
	3.0	11	9	»hydroxyglutamic acid», threonine, serine
	3.5		14	glutamic acid, aspartic acid, serine, *iso*leucine
	3.83 4.11 4.30	13.3		glutamic acid, serine
	2			glutamic acid (leucine), serine
	3.01		9	5 glutamic acid, 4 serine
	3.33		10	5 glutamic acid 1 *iso*leucine, 4 serine
37.0	3.56	9.3	10—11	glutamic acid, aspartic acid, *iso*leucine, serine
28.06	2.69	12.14		glutamic acid, *iso*leucine, serine
35.5	2.37	14.37	7	» » » »
	4	12.6	8	2 glutamic acid, 2 serine
33.45	3.20	9.94	10	3 glutamic acid, 3 *iso*leucine, 4 serine
33.12	3.35	9.84		
34.02	3.16	9.98		
33.31	3.30	9.96		

resistance to pepsin found for α-casein separated electrophoretically or obtained by other preparative methods, it is striking that in one case a high phosphorus content is combined with a pronounced resistance and in the other case with a higher sensibility to proteolysis. It must be borne in mind, however, that in the case of α-casein we are dealing with a fraction of the native casein and in the other case with a product obtained after enzymatic cleavage of the molecule.

Analytical data on phosphopeptones prepared by different authors are collected in Table 11.

AUTHOR'S EXPERIMENTS

The amino-acid composition of phosphopeptones from cow's casein now seems to be fairly well established. The analytical differences that still remain may be due to one or other of two causes, *viz.* variation of the composition of the peptides, or the presence of impurities in the

form of amino acids or lower peptides. The difficulties that may be encountered in removing amino acids from peptides are well illustrated by the recent work of EDMAN (1945) on hypertensin. The phosphopeptones, no doubt, are, however, characteristic end-products of the tryptic, *in vitro* hydrolysis of casein. It has also been suggested that they might be formed during the intestinal digestion of casein, and that they are resorbed without further breakdown. It is evident, however, that this can only be the case when the phosphopeptones are able to form salts soluble under the conditions prevailing in the intestines. So far, in most cases free phosphopeptone or salts of barium or heavy metals have been prepared. These salts have, of course, no physiological significance. The calcium or the alkali metal salts may, on the other hand, be important in this respect. As far as the author has found, no method for the preparation of the calcium salts seems to have been described.[1] A method has therefore been worked out for the preparation of calcium phosphopeptone in amounts sufficient for, *inter alia*, resorption experiments. Phosphopeptones from human casein have not hitherto been prepared. The action of phosphatase preparations on the course of the proteolysis of human casein, described in the previous chapter, indicates that phosphopeptones are also formed when human casein is digested. It was also to be expected that a greater part of the phosphorus of human casein should be found in the phosphopeptone, provided of course that the two phosphopeptones have similar properties. As will be described below, it is possible to prepare substances from human casein analogous to the phosphopeptones from cow's casein.

Preparation of phosphopeptones

The preparative method at first used was that described by DAMODARAN and RAMACHANDRAN. After completed peptic digestion these authors subject the insoluble residue of pseudonuclein to tryptic hydrolysis. Along those lines the present author has prepared a number of barium phosphopeptones from cow's casein, the elementary compositions of which agreed fairly well with those of DAMODARAN and RAMACHANDRAN. A description of a typical preparation according to this method follows:

200 g of cow's casein were suspended in 5 litres of 0.1 N sulphuric acid. The pH of the resulting mixture was 1.4. 5 g of pepsin (KAHLBAUM, D. A. B. 6) were added and digestion allowed to proceed for four days, when a further 5 g of pepsin were added and the pH of the digerate adjusted to 1.8 by addition of the appropriate amount of 0.1 N sulphuric acid. After digestion for a total period

[1] T. POSTERNAK and POLLACZEK have started their preparation of "phosphopeptone I and II" from a crude preparation of the calcium salt, apparently prepared by S. POSTERNAK. In the latter's publication (S. Posternak 1928) no experimental details are given, however, concerning the preparation or properties of the calcium salt.

of six days, the amino nitrogen[1] was 8.8 % of total nitrogen, after seven days it had risen to 11.4 %, and was then unchanged after eight days. 5 g of pepsin were again added, and the pH adjusted as described above. After digestion for a total period of 14 days the amino nitrogen was 15.6 % of the total nitrogen, and did not rise on further digestion. The precipitate of pseudonuclein was separated in the centrifuge and washed with water until free from sulphuric acid. The amount obtained had a dry weight of 50 g and contained 13.6 % N and 2.3 % P.

The pseudonuclein was suspended in one litre of water and 1 N sodium hydroxide added in small portions, stirring continually until all the pseudonuclein was dissolved and a pH of 8.3 was reached. 1 g of a commercial trypsin preparation (Difco 1/110) was added and digestion allowed to proceed at a temperature of 37° C for 24 hours, when 1 g more of the trypsin was added and the pH adjusted to 8.3. The cleavage was followed by means of formol titration. Constant values were obtained after four days' digestion.

The pH of the digerate was now adjusted to 4.6 by means of glacial acetic acid, and the mixture kept in the refrigerator overnight. The small precipitate formed was centrifuged off. To the residue was added a saturated solution of lead acetate until no more precipitation occurred. The precipitate was centrifuged off and suspended in one litre of water. Hydrogen sulphide was led in until all lead had been removed as lead sulphide. The acidic, water-clear solution of phosphopeptone was adjusted to a pH of 8.3 by means of 1 N sodium hydroxide, and redigested with 0.5 g of trypsin for three days. Precipitation by means of lead acetate etc. was carried out as above. To the clear solution obtained after removing the lead sulphide were added 6 g of barium acetate dissolved in a small amount of water. Barium hydroxide was added until the solution gave an alkaline reaction to litmus. The solution was rapidly filtered into a beaker containing glacial acetic acid. The pH was adjusted to 7 by means of barium hydroxide, and to the resulting solution was added an equal volume of 96 % ethanol. The resulting precipitate of barium phosphopeptone was washed 6 times with a total amount of 600 ml of 50 % ethanol, followed by two washings with a total amount of 200 ml of absolute ethanol, and finally by one with 100 ml of ether. The preparation was dried *in vacuo* over calcium chloride at a temperature of 40° C. Dry weight 3.24 g.

In this way three preparations (phosphopeptones Nos. C 1, C 2 and C 3) were made. The analytical figures are given in Table 12. As may also be seen from the table the yields were poor, however, (Damodaran and Ramachandran give no figures for the yields obtained), and attempts were therefore made to improve the method of preparation. It was found that if tryptic hydrolysis is performed on the whole peptic digest including the suspended precipitate of pseudonuclein, instead of on the latter only, the yield is considerably improved. This is the only possible method for the preparation of phosphopeptone from human casein, as no precipitate of pseudonuclein is obtained during the initial peptic hydrolysis. It was also found that better yields were obtained when tryptic digestion was carried out at a less alkaline reaction than in previous preparations (pH 7.0–7.5 instead of 8.0–8.5). This is probably due to the fact that commercial trypsin preparations contain considerable amounts of alkaline phosphatase and that the

[1] Determined according to the method of Van Slyke (1912).

activity of the latter is lower at the less alkaline pH. The sample of pancreatin (Parke, Davis & Co.) used for these digestions was analyzed with regard to its content of alkaline phosphatase. 100 ml of a 5 % solution showed an alkaline phosphatase activity of about 11 units. It was found that the activity was appreciably diminished when the pancreatin solution was acidified to a pH of about 2 and heated to 50° C for 30 minutes. After this treatment only a very faint activity persisted. The proteolytic activity was practically unchanged. The pepsin preparation used was free from acid phosphatases. In most cases the calcium salts of the phosphopeptones were prepared. A typical preparation is described below:

4 kg of casein prepared according to SANDELIN were suspended in 65 litres of distilled water. 100 ml of chloroform was added to check bacterial growth. 16 litres of 0.1 N sodium hydroxide were added slowly, with mechanical stirring. The casein dissolved and the pH of the resulting solution was 6.4. The casein solution was poured by instalments into 16 litres of 0.3 N hydrochloric acid. The resulting acid solution of casein had a pH of 2.1. 100 g of pepsin (Parke, Davis & Co. 1/3000) were now added and digestion allowed to proceed at a temperature of 37° C. After two days the pH, which was now about pH 3, was adjusted to pH 2 by addition of dilute hydrochloric acid, and another 100 g of pepsin added. This procedure was repeated once. Every day 100 ml of chloroform were added to the solution. After 12 days the amino nitrogen reached a constant value (about 15 % of total N), and digestion was therefore interrupted. The digerate was adjusted to pH 7.5 by addition of dilute sodium hydroxide, 20 g of pancreatin[1] were added, and the digestion continued at 37° C. After three days the pH was adjusted to the initial value and another 20 g of pancreatin added. After 8 days the amino nitrogen was constant (about 30 % of total N), and digestion was interrupted.

The digerate was adjusted to pH 4.6 by addition of 950 ml of glacial acetic acid. A small amount of precipitate was removed in the centrifuge and the phosphopeptone precipitated by addition of 1650 ml of a saturated solution of lead acetate. The resulting precipitate of lead phosphopeptone was allowed to settle down overnight. The supernatant clear liquid was then removed by suction and the precipitate finally freed from liquid in the centrifuge. The lead phosphopeptone was suspended in five litres of water and decomposed by means of hydrogen sulphide. The lead sulphide was centrifuged off and the solution of phosphopeptone freed from hydrogen sulphide by means of aeration. To the solution was then added 77 g of calcium acetate (in 10 % water solution) followed by saturated calcium hydroxide until faintly alkaline reaction. The pH was adjusted to about 7 by addition of acetic acid and, if not clear, the solution was centrifuged. 7.5 litres of 96 % ethanol was added with vigorous stirring. The precipitate of calcium phosphopeptone was collected in the centrifuge and washed with ethanol and ether in the usual manner. After drying for a week at a temperature of 40° the dry weight of the calcium phosphopeptone was 330 g. No redigestion was carried out.

Three preparations of calcium phosphopeptone from cow's casein and one from human casein have been carried out. In an analogous manner also one preparation of barium phosphopeptone from human casein was performed. The analytical data are given in Table 12.

[1] Phosphatase activity removed as described above.

Table 12. *Composition of phosphopeptones prepared by the author.*

Preparation	Method of preparation[1]	Amount of casein used	Yield g	Yield %	% of casein phosphorus in the phosphopeptone	C %	H %	N %	P %	S %	Ba[2] %	Ca %	N P (atomic ratio)	Amino N in % of total N
From cow's casein														
C 1	DR	200	3.24	1.6	10	21.58	2.68	5.95	4.85	0	35.48	0	2.72	13
C 2	DR	660	8.50	1.3	10	20.44	2.76	5.60	5.98	0	38.50	0	2.07	14
C 3	DR	1000	3.6	0.36	2	19.83	2.76	4.97	5.05	0	37.76	0	2.18	—
C 4	M	25	1.7	6.8	45	23.71	3.06	7.42	5.34	0	35.20	0	3.08	9
C 5	M	1300	120.5	9.3	62	23.43	3.38	8.21	5.37	0	31.08	0	3.39	11
C 6	M	700	49.7	7.1	54	32.91	4.31	9.64	6.13	0		9.00	3.48	—
C 7	M	4060	330	8.1	57	34.69	4.55	10.30	5.62	0		9.96	4.07	10
From human casein														
H 1	M	25	1.2	4.8	43			6.57	4.28	0	33.6	0	3.40	11
H 2	M	78	4.5	5.8	67	34.05	4.38	5.80	5.46	0		11.25	2.35	

[1] DR = Damodaran and Ramachandran (1941),
M = author's modification.

[2] Barium vas determined gravimetrically as barium sulfate after hydrolysis with hydrochloric acid.

Digestion experiment No. 29.

Substrate: 2 g of calcium phosphopeptone C 7 (from cow's casein) and H 2 (from human casein) respectively, 2 ml of an 1 % solution of magnesium chloride and 98 ml of distilled water.
Proteolytic enzyme: pancreatin (Parke Davis & Co.) 0.1 %.
Phosphatase: preparation from infant's intestines.
Hydrogen ion concentration: pH 8.1.
Temperature: 37° C.

Pancreatin + active phosphatase			Pancreatin + heat inactivated phosphatase		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
		Calcium phosphopeptone C 7			
2	0.82	0.00	2	0.82	0.00
60	1.35	0.53	60	1.30	0.48
120	1.40	0.58	120	1.38	0.56
180	1.42	0.60	185	1.42	0.60
Immediately after this titration 0.3 ml of the phosphatase solution was added to 30 ml of the digerate			Immediately after this titration 0.3 ml of heat inactivated phosphatase solution was added to 30 ml of the digerate		
185	1.41	0.60	190	1.42	0.60
230	1.47	0.66	230	1.45	0.63
270	1.52	0.71	270	1.48	0.66
360	1.68	0.87	360	1.48	0.66
		Calcium phosphopeptone H 2			
2	0.63	0.00	2	0.60	0.00
60	0.72	0.09	60	0.70	0.10
120	0.77	0.14	120	0.76	0.16
190	0.86	0.23	195	0.82	0.22
Immediately after this titration 0.3 ml of the phosphatase solution was added to 30 ml of the digerate			Immediately after this titration 0.3 ml of heat inactivated phosphatase solution was added to 30 ml of the digerate		
195	0.84	0.23	200	0.83	0.22
230	0.90	0.29	230	0.88	0.27
270	0.96	0.35	270	0.92	0.31
360	0.98	0.37	360	0.96	0.35

Properties of calcium phosphopeptone

The calcium phosphopeptones from cow's and human casein are obtained in the form of fine white powders which are very soluble in water (50 g of cow's casein phosphopeptone readily dissolves in 100 ml of wa-

Digestion experiment No. 30.

Substrate: 2 g of calcium phosphopeptone C 7 (from cow's casein) and H 2 (from human casein) respectively, 2 ml of an 1 % solution of magnesium chloride and 98 ml of distilled water.
Proteolytic enzyme: pancreatin (Parke Davis & Co.) 0.1 %.
Phosphatase: preparation from calf's intestines.
Hydrogen ion concentration: pH 8.1.
Temperature: 37° C.

Pancreatin + active phosphatase			Pancreatin + heat inactivated phosphatase		
Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes	Time of enzyme action, minutes	Formol titration 0.05 N sodium hydroxide, ml	Difference from value at two minutes
		Calcium phosphopeptone C 7			
2	0.72	0.00	2	0.74	0.00
60	1.06	0.34	60	1.08	0.34
120	1.26	0.54	120	1.24	0.50
180	1.30	0.58	185	1.32	0.58
Immediately after this titration 0.3 ml of the phosphatase solution was added to 30 ml of the digerate			Immediately after this titration 0.3 ml of heat inactivated phosphatase solution was added to 30 ml of the digerate		
185	3.46	0.58	190	3.44	0.58
260	3.64	0.76	260	3.52	0.66
300	3.75	0.87	300	3.60	0.74
360	3.80	0.92	360	3.66	0.80
		Calcium phosphopeptone H 2			
2	0.50	0.00	2	0.50	0.00
60	0.64	0.14	60	0.64	0.14
120	0.66	0.16	120	0.70	0.20
190	0.72	0.22	195	0.74	0.24
Immediately after this titration 0.3 ml of the phosphatase solution was added to 30 ml of the digerate			Immediately after this titration 0.3 ml of heat inactivated phosphatase solution was added to 30 ml of the digerate		
195	2.68	0.22	200	2.72	0.24
260	2.72	0.26	260	2.76	0.28
300	2.90	0.44	300	2.88	0.40
360	2.92	0.46	360	2.84	0.36

ter). Such concentrated solutions have a brown tinge and are highly viscous. The water solutions show a weak alkaline reaction. Most of the preparations obtained have been analyzed for carbon, hydrogen, nitrogen, phosphorus, sulphur and calcium (see Table 12). It was also found that none of the preparations contained lead. Amino nitrogen was determined on one of the preparations from cow's casein, using the method of Van Slyke.

The stability of the phosphopeptones towards peptic hydrolysis was tested by digesting a 2 %. solution of calcium phosphopeptone (C 6) for 120 minutes at pH 2 with 0.05 %. pepsin (Parke, Davis & Co.). 11.70 ml of 0.05 N sodium hydroxide were required in the formol titration of 5 ml of digerate, as against 11.66 ml two minutes after adding the enzyme. A similar negative result was obtained in an experiment on phosphopeptone from human casein (H 2).

The action of trypsin and phosphatase from calf's and infant's intestines on calcium phosphopeptone from human and cow's casein has been studied in digestion experiments Nos. 29 and 30.

Discussion and Summary of Chapter IV

According to the method of Damodaran and Ramachandran (1941) three preparations of barium phosphopeptone from cow's casein have been carried out. On analysis it is found (Table 12) that the nitrogen values are slightly lower and the phosphorus values slightly higher than those of the Indian authors. Two preparations of barium phosphopeptone were made by a modification of the method mentioned above. In this case the tryptic digestion was not performed on the pseudonuclein only, but on the whole of the peptic digest, and care was also taken to avoid undue phosphatase activity during the tryptic digestion. A considerably greater part of the total casein phosphorus was in this way obtained in the form of phosphopeptone. The N P ratio of the product was higher, however, than in the preparations according to the original method, indicating that the breakdown was less complete. This is also indicated by the values for amino nitrogen. Barium phosphopeptone was also prepared from human casein according to the modified method. The nitrogen and phosphorus values for this preparation agree rather well with the corresponding values for cow's casein phosphopeptone obtained by Damodaran and Ramachandran (cf. Table 11). The amino nitrogen was also of the same order of magnitude. The calcium salt of phosphopeptone from cow's casein has been prepared in large quantities without difficulty, two different preparations (C 6 and C 7) showing approximately the same elementary composition. It should perhaps be pointed out that the chief purpose was not to prepare products of constant elementary composition but rather to obtain sufficient amounts of calcium phosphopeptone for resorption experiments. The N·P ratios as also the amino nitrogen value of 10 % for C 7 indicate that proteolysis has not been complete. Also from human casein, phosphopeptone has been prepared in the form of calcium salt (H 2). Table 11 shows the analytical figures. The N P ratio was rather low (2.35) and the amount obtained contained a greater part of the total casein phosphorus than any of the preparations from cow's casein.

Calcium phosphopeptones from both human and cow's casein show a high degree of resistance to pepsin but are not stable against the commercial trypsin preparation used. This may be due to the presence of alkaline phosphatase. Addition of phosphatase from human intestines increases the rate of hydrolysis in case of phosphopeptone from cow's casein but there seems to be a very much smaller effect in the case of calcium phosphopeptone from human casein. Phosphatase from calf's intestines causes about the same increase in proteolysis for both human and cow's calcium phosphopeptone (cf. digestion experiments Nos. 29 and 30). Owing to the small quantities of phosphopeptone from human casein available it has not yet been possible to repeat these experiments.

The review of the literature given on page 129 shows that a large number of compounds of very different composition have been isolated from peptic digestion of cow's casein. The initial material used for the preparations has been the insoluble residue of pseudonuclein or the part of the digests remaining after removal of the latter. In spite of their different compositions most of the substances described have in common had a comparatively high content of organically bound phosphorus. It has also been found that the composition of the products obtained is altered on further digestion. It appears very unlikely, therefore, that these compounds are physiological end-products of the intestinal digestion of casein. This might be the case, however, with the enzyme-resistant phosphopeptones isolated from tryptic digests of cow's casein. These compounds contain a large part (about 50 %) of the phosphorus of the casein from which they are derived. It seems natural to suggest, therefore, that these compounds might be of importance for the phosphorus uptake of the organism, and, as they are sensitive towards phosphatases, that they might be of special importance for the formation of bone. This was suggested by Rimington (1927, b). As shown in the present work enzyme-resistant breakdown products can be prepared from human casein also. In one of the author's preparations, the amount of phosphopeptone obtained contained about 70 % of the total casein phosphorus, which is considerably more than in any of the preparations from cow's casein.

It thus appears that the end result of the *in vitro* digestion of both human and cow's casein with pepsin and with the proteolytic enzymes present in commercial trypsin preparations is not only amino acids but also certain peptides which are protected against further proteolytic action by being esterified with phosphoric acid. The rôle of these phosphopeptones in nutrition is of course a question of very great importance. A point of special interest is whether there are quantitative or qualitative differences in the phosphopeptones obtained from the digestion of human and cow's casein respectively and, if such differences are present, whether they can contribute to the known differences between human and cow's milk used as infant nourishment. So far,

it is not known whether phosphopeptones are also an end product during digestion under physiological conditions in the intestines, or if the breakdown goes further in this case. An essential point is to what degree the organically bound phosphorus is liberated from the phosphopeptone by the action of intestinal phosphatases. The action of the latter depends also on the intestinal pH. Some preliminary experiments by the present author indicate that phosphopeptone from human casein is comparatively resistant, especially vis-à-vis phosphatase from infants' intestines. Phosphopeptone from cow's casein, however, seems to be more susceptible to the action of human intestinal phosphatase. Phosphopeptone from human casein is more resistant than that from cow's casein also to the mixture of different enzymes present in pancreatin. The activity of the alkaline intestinal phosphatases is greatest at about pH 8—9, and the liberation of organically bound phosphorus from the phosphopeptones may therefore be expected to proceed at a lower rate when the reaction is neutral or slightly acid. It is of interest in this connection that several investigations carried out on faeces (*e. g.* Bosworth *et al.* 1922), seem to indicate that the intestinal reaction of breast-fed infants is on the whole more acid than that of infants fed with cow's milk. It thus appears possible, at least in the case of breast-fed infants, that the intestinal digestion of casein might result in the formation of enzyme-resistant peptides of the phosphopeptone type. For resorption of phosphopeptone thus formed it is of course necessary that the alkali metal and the alkaline earth metal salts of phosphopeptones should be soluble. As has been pointed out above, this is actually the case. Previously, in nearly all cases the phosphopeptone from cow's casein has been obtained as barium salt or as salts of heavy metals and, in some cases, as sodium salt or as free acid. Under physiological conditions, the calcium salt prepared and studied in the present work may well exist under conditions prevailing in the intestines. This salt contains calcium and phosphorus in considerable amounts, and is very soluble. To the organism, it therefore represents a convenient form of calcium and organically bound phosphorus. It is not necessary to consider compounds of this type as being well defined chemical substances. On the contrary it is more likely that they may have somewhat different structure with regard to amino acid composition and molecular weight, depending on variations in salt concentration and enzyme activity and other conditions which might vary during the physiological digestion. The different amino acid composition reported by different authors for phosphopeptones from cow's casein point in this direction. A definite understanding of the nutritional rôle of these compounds can only be obtained through resorption studies on infants. The amounts of calcium phosphopeptone necessary for such studies are quite considerable. The preparative method described in this paper allows a fairly easy preparation of

phosphopeptones in quantity as yields of about 300 g calcium phosphopeptone from 5 kg of casein have been obtained.

To sum up, the well known fact that feeding with human milk leads to better calcium and phosphorus utilization than feeding with cow's milk[1] may be explained by the following joint causes:

1. A larger part of the phosphorus of human casein is transformed into phosphopeptones through the action of proteolytic enzymes of the intestinal tract.
2. The more constantly acid reaction of the intestinal content of the breast-fed infants, which protects the phosphopeptones from the action of alkaline phosphatases.
3. The relatively small susceptibility of phosphopeptones from human casein to the intestinal phosphatase of infants, even at the optimal reaction of alkaline phosphatases.

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[1] Cf. *e. g.* Wang, Witt and Felcher (1924), Telfer (1930), Witt (1932) and Icy G. Macy (1932).

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